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**Surveillance et épidémiologie des fièvres hémorragiques
virales (FHV): identification de l'émergence, de la
séroprévalence et des facteurs de risque des FHV en
Ouganda**

Présentée par Trevor SHOEMAKER

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Devant le jury composé de

Martine PEETERS, Directeur de Recherche, IRD, Montpellier

Jean-Claude MANUGUERRA, Directeur de Recherche, Institut Pasteur

Frederick ARNAUD, Directeur d'Etudes Cumulant, EPHE

Raphaëlle METRAS, Chargée de Recherche Classe Normale, Inserm

Chiara POLETO, Chargée de Recherche Classe Normale, Inserm

Catherine MOULIA, Professeur des Universités, Université de Montpellier

Mathieu SICARD, Professeur des Universités, Université de Montpellier

Thierry LEFRANÇOIS, Chercheur, CIRAD

Présidente du Jury

Rapporteur

Rapporteur

Examineur

Examineur

Examineur

Examineur

Directeur du Thèse



**UNIVERSITÉ
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Dedication

To my family

Melissa, Annabelle, Caiden and Wesley

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Résumé de thèse

Introduction

Les fièvres hémorragiques virales (FHV) constituent un groupe de maladies causées principalement par des virus à ARN. Ils infectent à la fois les humains et les animaux (zoonotiques) et se caractérisent cliniquement par une forte fièvre et des symptômes hémorragiques parfois graves, en particulier aux stades ultimes de la maladie. Les virus des genres *Ebolavirus* et *Marburgvirus*, de la famille *Filoviridae*, sont connus pour provoquer des fièvres hémorragiques sous leur forme «classique». D'autres familles de virus, comme les *Phenuiviridae* (virus de la fièvre de la Vallée du Rift (FVR)), *Nairoviridae* (le virus de la fièvre hémorragique de Crimée-Congo (FHCC)), et *Arenaviridae* (virus de Lassa) font aussi partie des FHV.

Les manifestations cliniques précoces des FHV incluent maux de tête, fièvre, malaises, anorexie, arthralgie, et divers degrés de sévérité de nausées, vomissements et diarrhée, évoluant ensuite en hémorragies externes ou internes, en insuffisance rénale et en état de choc. Les patients infectés par des FHV présentent ces signes avec différents degrés de gravité et ne développent pas tous un syndrome hémorragique classique. Au début de la progression d'une infection à FHV chez l'homme, la plupart des cas se présentent dans un établissement de soins avec des symptômes compatibles avec d'autres infections tropicales plus courantes telles que le paludisme, la typhoïde ou les rickettsioses. Ces symptômes peu spécifiques rendent le diagnostic différentiel des FHV difficile pour le clinicien.

Toutes les FHV sont des zoonoses et certaines peuvent être transmises par des arthropodes vecteurs tels que le virus de la FHCC et le virus de la FVR, et peuvent avoir un hôte zoonotique, animal ou réservoir, tel que le bétail. Les filovirus, comme *Ebolavirus*, sont transmis par contact étroit avec une personne infectée ou ses fluides corporels infectés, ou par contact direct ou indirect avec son hôte réservoir naturel. L'hôte réservoir du filovirus *Marburgvirus* a été identifié comme étant la chauve-souris égyptienne, *Rousettus aegyptiacus*. Bien que les virus à l'origine des FHV soient répartis dans le monde entier, ils sont limités par la distribution de leurs espèces hôtes réservoirs.

Les épidémies de FHV, notamment la maladie à virus Ebola, la maladie à virus de Marburg (MVD), et la fièvre de la vallée du Rift (FVR), ont été bien documentées dans toute l'Afrique subsaharienne et sont à l'origine d'une morbidité et d'une mortalité importantes, ainsi que de pertes socio-économiques considérables. Vingt-sept (27) foyers confirmés d'infection par le virus Ebola ont été signalés depuis la première identification dans l'ex-Zaïre (aujourd'hui République Démocratique du Congo, RDC) en 1976. De plus, 13 foyers de MVD ont été confirmés depuis sa première identification en Allemagne en 1967. Les foyers les plus importants ont été les foyers EVD d'Ebola Soudan en Ouganda en 2000, et pour Ebola Zaire l'Afrique de l'Ouest (plusieurs pays affectés) en 2014-15 et plus récemment la RDC, en 2018 et 2019. En plus de l'impact sanitaire, l'impact économique de l'épidémie d'Ebola en Afrique de l'Ouest est estimé à plus de 2,2 milliards de dollars américains (USD) du PIB, incluant la perte de l'investissement étranger, le déclin de la croissance du secteur privé, une baisse des productions agricoles et des échanges commerciaux. Les coûts pour les pays touchés et la communauté internationale pour répondre et contrôler l'épidémie a dépassé 3,6 milliards de dollars.

De vastes flambées de FVR à travers l'Afrique ont également provoqué des épidémies au sein des populations humaines. Les pertes socio-économiques les plus importantes sont dues à la morbidité et à la mortalité du bétail. En raison de la nature écologique, zoonotique et vectorielle de la FVR, les épidémies ont tendance à toucher principalement les régions à forte population animale et les pays où l'économie locale est fortement dépendante du commerce de l'élevage et de l'agriculture. Parmi les principales épidémies de FVR enregistrées depuis 1930, l'impact socio-économique varie de 7 à 470 millions de dollars, représentant des pertes de plus de 5 % du PIB. Les principaux coûts associés aux épidémies de FVR sont dus aux impacts sur le commerce local et international, aux pertes de l'industrie de l'élevage et aux dépenses de santé publique et de soins de santé.

En raison de la menace persistante et croissante des FHV, tels que la maladie à virus Ebola, la MVD et la FVR, et de leur effets néfastes importants sur la santé, le bien-être et les économies des pays affectés, l'OMS a lancé le

programme « Blueprint » pour la recherche et le développement concernant les agents pathogènes prioritaires. Blueprint R&D est une stratégie globale et un plan de préparation qui fournit un cadre permettant de hiérarchiser les domaines et les activités de recherche afin d'accélérer le développement collaboratif de contremesures médicales (MCM), notamment les outils diagnostics, les traitements et les vaccins contre ces agents pathogènes prioritaires. En visant à accélérer le développement et la mise en œuvre de ces mesures préventives, elles peuvent être utilisées pour sauver des vies et éviter ou limiter de futures épidémies de grande ampleur. Les filovirus et la FVR sont inclus dans le programme Blueprint de l'OMS.

Pour développer des contremesures pour améliorer le contrôle et le confinement des épidémies de FHV, il est tout aussi important de comprendre les facteurs épidémiologiques et leurs risques associés, ainsi que de déterminer les caractéristiques moléculaires et cliniques des agents pathogènes qui contribuent à leur émergence. Bien que beaucoup de progrès et de connaissances aient déjà contribué au programme Blueprint, il reste de nombreuses lacunes dans la connaissance de certains des agents pathogènes prioritaires. Une compréhension plus complète de leurs facteurs écologiques, épidémiologiques, pathogéniques et génomiques nous permettra d'appréhender leur potentiel d'émergence, d'améliorer la surveillance humaine et zoonotique, de réaliser des tests de diagnostic plus sensibles et plus spécifiques, et de caractériser leur incidence et leur prévalence dans les zones d'endémie afin de mettre en œuvre des stratégies de contrôle et de prévention adaptés.

L'objectif principal de cette thèse est d'étudier les caractéristiques scientifiques et épidémiologiques des FHV, avec comme application d'étude l'infection au virus Ebola et au virus de la fièvre de la Vallée du Rift, dans le but d'apporter des connaissances qui permettront d'améliorer la surveillance, la détection précoce, les enquêtes épidémiologiques, le contrôle et la prévention de ces maladies. Cet objectif principal est atteint en réalisant la série d'objectifs secondaires suivants.

1. Utiliser les techniques moléculaires et sérologiques existantes pour identifier et caractériser génétiquement le premier foyer de fièvre de la vallée du Rift (FVR) identifié en dehors du continent africain
2. Développer et valider une nouvelle analyse RT-PCR quantitative sensible et à spectre large pour la détection rapide de la FVR
3. Concevoir et mettre en œuvre un système de surveillance complet des fièvres hémorragiques virales (FHV) en Ouganda afin de détecter, d'intervenir et de contrôler rapidement les épidémies de FHV
4. Décrire l'efficacité du système de surveillance FHV en Ouganda et quantifier les principaux indicateurs de réponse et de contrôle
5. Effectuer une comparaison génétique des isolats de filovirus obtenus à partir de la surveillance FHV en Ouganda et analyser les facteurs de virulence génétiques pouvant contribuer à leur ré-émergence
6. Mettre en place des enquêtes de terrain afin de caractériser la ré-émergence de la FVR en Ouganda
 - a. Réaliser une enquête épidémiologique complète et une analyse phylogénétique des isolats de virus de la FVR afin d'identifier la ou les sources de l'émergence
 - b. Déterminer la séroprévalence de la FVR chez l'homme et le bétail et identifier les facteurs de risque associés à la FVR dans certaines régions de l'Ouganda
 - c. Décrire les connaissances, attitudes et pratiques (CAP) associées à la FVR en Ouganda

Méthodes / Résultats

Objectif 1: Utiliser les techniques moléculaires et sérologiques existantes pour identifier et caractériser génétiquement le premier foyer de fièvre de la Vallée du Rift (FVR) identifié en dehors du continent africain (Manuscrit 1)

Le premier manuscrit décrit la première identification et caractérisation génétique de la FVR en dehors de l'Afrique. Il s'agissait d'un événement important démontrant le potentiel de propagation de la maladie dans d'autres régions. Nous avons effectué des tests de diagnostic sur plusieurs échantillons humains prélevés lors de

cette émergence pour caractériser les cas de FVR. Le diagnostic en laboratoire utilisant la RT-PCR conventionnelle et les techniques sérologiques disponibles à l'époque, comprenait la détection génétique du virus et la caractérisation à partir d'échantillons cliniques par réaction en chaîne par polymérase par transcription inverse (RT-PCR), en plus des tests sérologiques et l'isolement viral.

De plus, nous avons effectué l'analyse phylogénétique des segments S, M et L du génome ARN. Les analyses génétiques ont révélé une faible variation génétique. Les virus de l'Arabie Saoudite et du Yémen étaient presque identiques à ceux associés aux précédentes épidémies de FVR en Afrique de l'Est, en particulier aux virus responsables de l'épidémie majeure de 1997–98 de la corne de l'Afrique. Ces résultats étaient cohérents avec l'introduction récente du virus de la FVR dans l'Arabie d'Afrique de l'Est.

Objectif 2: Développer et valider une nouvelle analyse RT-PCR quantitative sensible et largement réactive pour la FVR pour la détection rapide en laboratoire de la FVR (Manuscrit 2).

Ce travail visait à améliorer les tests de diagnostic de la FVR existants et à développer un test de diagnostic moléculaire par RT-PCR en temps réel. Le manuscrit associé détaille le développement d'un test RT-PCR (Quantitative-RT-PCR) quantitatif en temps réel avec une conservation élevée des nucléotides des régions d'amorce et de sonde pour un total de 40 souches de FVR qui, lorsqu'elles sont couplées à des méthodes d'extraction à haut débit, permettent l'identification rapide des patients et animaux infectés. Cet essai a démontré sa capacité à détecter de manière extrêmement sensible et efficace le virus de la FVR dans du matériel biologique humain en utilisant des échantillons de patients archivés, prélevés en Arabie Saoudite lors de la flambée de FVR au cours de l'année 2000, décrite précédemment. La détection rapide et à haut débit de l'infection humaine ou animale par le virus de la FVR était une première étape cruciale dans l'identification et le contrôle éventuel de cet agent pathogène lors de futures épidémies.

Objectif 3: Concevoir et mettre en œuvre un système de surveillance complet des fièvres hémorragiques virales (FVH) en Ouganda afin de détecter, d'intervenir et de contrôler rapidement les épidémies de FVH (Manuscrit 3)

Ce troisième manuscrit fait suite à la mise en place du système national de surveillance des FHV en Ouganda et décrit la première émergence de FHV, une infection isolée à Ebolavirus-Soudan (SUDV). Cette première détection virale a été suivie dans les deux années suivantes de la détection, la confirmation et l'étude de 4 épidémies supplémentaires causée par des filovirus. Parmi ceux-ci figuraient deux épidémies à Ebolavirus-Soudan en Ouganda, une épidémie de Marburgvirus (MVD) en Ouganda et une confirmation d'infection à Bundibugyo Ebolavirus (BDBV) à l'Est de la République démocratique du Congo (RDC). L'augmentation du nombre d'émergences de filovirus a suscité l'inquiétude de la communauté internationale quant à d'éventuelles modifications génétiques pouvant rendre ces souches plus virulentes, ou les ré-émergences plus fréquentes.

Le programme national de surveillance et de laboratoire des FHV en Ouganda a été lancé en 2010 en collaboration avec UVRI (Uganda Viral Research Institute), le ministère de la Santé ougandais (Mds) et la Viral Special Pathogens Branch (VSPB) du Centers for Disease Control and Prevention (CDC). Ce système national de surveillance, une « première du genre », établie en Ouganda, a été mis en place afin d'améliorer la surveillance intégrée des maladies et leur réponse (SIMR) en permettant de détecter rapidement, diagnostiquer, signaler l'émergence de FHV, ainsi que d'autres zoonoses. Le programme visait également à élargir et à améliorer les tests de diagnostic en laboratoire pour les FHV en Ouganda, à améliorer la détection des incidents en améliorant la reconnaissance clinique et à utiliser les données générées pour mieux éclairer les efforts de prise de décision et de lutte contre la maladie au niveau national.

Ce programme a été mis en œuvre grâce à la création de plusieurs sites de surveillance sentinelles directement reliés au laboratoire nouvellement créé à l'UVRI, à Entebbe (Ouganda). La formation du personnel des hôpitaux régionaux et des bureaux de santé de district comprenait la collecte de données épidémiologiques, la surveillance des FHV, et l'identification des patients suspects. Les éléments clés du programme ont été l'élaboration d'une définition de cas normalisée et d'un formulaire de notification des cas spécifique aux FHV.

Chaque site peut signaler un cas suspect, collecter et expédier en toute sécurité les échantillons à tester à l'UVRI et à notifier au ministère de la santé. Le programme comptait initialement 6 sites de surveillance sentinelle. Il en compte désormais plus de 20.

Objectif 4: Décrire l'efficacité du système de surveillance des FHV en Ouganda et quantifier les principaux indicateurs de réponse et de contrôle (Manuscrit 4).

Suite à la mise en œuvre du réseau de surveillance des FVH en 2010, et à la détection de 11 flambées supplémentaires indépendantes de FVH entre 2011 et 2017, ce manuscrit décrit la performance du système de surveillance sur la détection rapide et la réponse aux foyers de FHV. Suite à la mise en place du système, le nombre de détections d'épidémies a été multiplié par cinq par rapport aux 10 années précédentes. La taille et l'étendue de chaque émergence ont également été considérablement réduites, le délai entre la notification clinique initiale et la confirmation en laboratoire étant passé d'une moyenne de deux semaines avant 2010 à deux jours et demi. Cette détection précoce conduisant à une réponse plus rapide a entraîné une diminution significative de l'intensité globale (p -value = 0,001) et de la durée (p -value <0,001) des épidémies de FHV, ainsi qu'une réduction significative de la morbidité (p = 0,001) et de la mortalité (p = 0,01). Cela souligne la nécessité de la mise en place de moyens adéquates de façon continue pour les programmes de surveillance de santé publique et de lutte contre les zoonoses émergentes. Grâce à la mise en œuvre réussie de ce programme, l'Ouganda a détecté et confirmé en laboratoire de multiples épidémies de cas de fièvres hémorragiques virales. Cela comprenait la confirmation d'Ebola (Bundibugyo et Soudan) en 2011, de la fièvre hémorragique de Marburg en 2011-2014, et de la fièvre hémorragique de Crimée-Congo en 2013, 2015 et 2017. Cela a aussi permis de confirmer la première épidémie en 50 ans de FVR humaine en Ouganda (en 2016). La FVR continue d'être périodiquement détectée. L'Ouganda vient également de confirmer ses premiers cas importés d'Ebolavirus (Ebola du Zaïre) en provenance de la République démocratique du Congo (RDC) en 2019.

Objectif 5 : Effectuer une comparaison génétique des isolats de filovirus obtenus à partir de la surveillance des FHV en Ouganda et analyser les facteurs de virulence génétiques pouvant contribuer à leur ré-émergence (Manuscrit 5).

Ce manuscrit compare les séquences génétiques de souches épidémiques nouvellement identifiées aux souches épidémiques de filovirus historiques. Il est conclu qu'il n'y a pas eu de modifications génétiques significatives des nouvelles souches de filovirus qui auraient pu être à l'origine d'une augmentation de la virulence ou de la pathogénicité, entraînant l'augmentation observée de la fréquence des épidémies.

Entre juillet et octobre 2012, des cas d'alerte de FHV ont été identifiées en Ouganda ou signalées en République Démocratique du Congo (RDC). Des échantillons ont été reçus par le programme de surveillance des FHV. Quatre émergences indépendantes de filovirus ont d'abord été identifiées et confirmées en testant des échantillons cliniques par capture d'antigène, IgM ELISA et / ou qRT-PCR. Des échantillons de cas d'alerte FHV dans le district de Kibaale dans l'ouest de l'Ouganda ont été reçus au laboratoire CDC/UVRI en juillet 2012. Des tests moléculaires différentiels sur les échantillons cliniques ont identifié une épidémie à virus Ebola associée au SUDV. L'analyse génomique a révélé une identité de séquence d'environ 99,9% entre les génomes de virus détectés dans les quatre échantillons (sérum de cas aigus).

En août 2012, des échantillons provenant de cas d'alerte FHV dans la zone d'Isiro, dans la province orientale du nord-est de la RDC, ont été envoyés à l'UVRI pour des tests FHV. Le séquençage complet des génomes a confirmé la présence de BDBV dans les échantillons cliniques et a montré que ces virus étaient à 98,6% identiques à l'original BDBV isolé dans le district de Bundibugyo de l'Ouest de l'Ouganda en 2007.

En octobre 2012, des échantillons de cas d'alerte VHF reçus dans les districts de Kabale et d'Ibanda, dans le sud-ouest du pays, ont été analysés. Des génomes viraux complets prélevés dans des échantillons de sérum provenant d'un cas aigu de chaque groupe montraient des séquences presque identiques (99,9%), suggérant une chaîne de transmission interhumaine. De plus, ces séquences de génome viral étaient très similaires (99,3%)

à deux isolats de MARV précédemment trouvés dans *Rousettus aegyptiacus* les chauves - souris capturées en 2008 et 2009 non loin dans la Python cave dans le parc national Queen Elizabeth.

En novembre 2012, le laboratoire UVRI/CDC a reçu des échantillons de cas d'alerte de FHV dans le district de Luwero, qui l'ont identifiée comme une autre émergence de SUDV. Au cours des semaines qui ont suivi, six cas ont été confirmés dans les districts relativement proches de Luwero: Jinja et Nakasongola. Les gènes NP des détectées dans ces trois échantillons cliniques étaient presque identique (environ 100%) suggérant aussi une seule chaîne de transmission inter-humaine. Enfin, la séquence génomique complète obtenue à partir d'un isolat viral a montré que l'identité la plus proche était celle de la souche SUDV Gulu isolée en Ouganda en 2000.

Objectif 6: Mettre en place des enquêtes de terrain afin de caractériser la ré-émergence de la FVR en Ouganda

Les trois manuscrits finaux (6, 7 et 8) se concentrent sur l'identification, l'investigation et la caractérisation du virus de la fièvre de la Vallée du Rift en Ouganda. En mars 2016, le programme de surveillance a confirmé la première émergence de FVR en Ouganda depuis 1968.

a. Réaliser une enquête épidémiologique complète et une analyse phylogénétique des isolats de virus de la FVR afin d'identifier la ou les sources de l'émergence (Manuscrit 6).

En 2016, un total de 4 cas de FVR confirmés en laboratoire et 2 décès probables ont été identifiés dans le district de Kabale. Une équipe multidisciplinaire a mené une enquête de terrain sur les lieux des foyers d'émergence dans les deux jours suivant la confirmation initiale en laboratoire. Cette enquête a consisté à collecter des échantillons humains, de bétail et de vecteurs afin d'identifier d'autres cas et d'explorer les sources possibles d'infection. Les échantillons de laboratoire recueillis auprès de 19 membres de la famille et de la communauté appartenant aux cas confirmés et probables ont révélé deux autres cas de FVR séropositifs, montrant que le virus avait circulé avant la détection des foyers en questions. Par ailleurs, un total de 83 échantillons de bétail ont été recueillis, dont 8 (9,6%) ont montré des signes d'infection par le virus de la FVR. Un total de 6 genres différents de moustiques vecteurs, représentant 33 espèces et sous-espèces, ont été collectés à proximité des emplacements des cas confirmés et probables. Le virus de la FVR a été détecté dans 3 des 298 (1%) pools testés par RT-PCR, suggérant une transmission active par les vecteurs au moment de l'émergence.

b. Déterminer la séroprévalence de la FVR chez l'homme et le bétail et identifier les facteurs de risque associés à la FVR dans certaines régions de l'Ouganda (Manuscrit 7)

Suite à cette première détection de RVFV en 2016, nous avons lancé une étude visant à évaluer la séroprévalence du virus de la FVR chez l'homme et chez les animaux vivant dans le district de Kabale et ses environs. Les objectifs de l'étude étaient : de déterminer la séroprévalence de la FVR chez les humains et les animaux dans Kabale et les districts environnants, d'identifier les facteurs de risque et les zones à risque accru de FVR, de déterminer si la FVR circulait toujours, et d'identifier les cas de FVR non diagnostiqués qui pouvaient être liés à l'émergence de 2016. Nous avons trouvé des preuves de séropositivité FVR chez 13% des humains et des animaux échantillonnés. Notre étude a également montré que les bouchers et ceux qui manipulaient de la viande crue étaient plus susceptibles d'être séropositifs FVR. Bien qu'aucun cas de maladie n'ait été détecté en Ouganda de 1968 à mars 2016, notre étude suggère que le FVR avait circulé sans être détectée chez l'homme et les animaux vivant dans, et autour du district de Kabale. Chez l'homme, la séropositivité était associée à l'activité professionnelle, ce qui suggère que le principal mode de transmission du virus FVR à l'homme dans le district de Kabale serait par contact avec du sang animal ou des fluides corporels infectieux.

c. Décrire les connaissances, attitudes et pratiques (CAP) associées à la FVR en Ouganda (Manuscrit 8).

Après le diagnostic des cas de FVR en mars 2016 dans le district de Kabale, dans le sud du pays, nous avons mené une enquête sur les connaissances, les attitudes et les pratiques (CAP) afin d'identifier les lacunes dans les connaissances et les comportements à risque liés à la FVR. Une équipe multidisciplinaire a interrogé 657

membres de la communauté, y compris des ouvriers d'abattoirs, dans le district de Kabale et ses environs. La plupart des participants (90%) connaissaient bien la FVR et la plupart (77%) ont cité la radio comme principale source d'information. Une plus grande proportion d'agriculteurs (68%), d'éleveurs (79%) et de bouchers (88%) pensaient qu'ils risquaient de contracter la FVR par rapport aux personnes occupant d'autres professions (60%, p-value <0,01). La plupart des éleveurs et des bouchers (36% et 51% respectivement) connaissaient les symptômes de la FVR chez les animaux par rapport à ceux des autres professions (30%, p-value <0,01). Les connaissances, les attitudes et les pratiques concernant la FVR dans le district de Kabale en Ouganda pourraient être améliorées grâce à des efforts d'éducation visant des populations spécifiques.

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Discussion / Perspectives

Les manuscrits décrits ci-dessus et qui constituent l'ensemble des travaux scientifiques et épidémiologiques présentés dans cette thèse, fournissent une description complète de la manière dont les outils d'analyse moléculaire et génétique initialement utilisés pour la détection des épidémies de FHV peuvent être développés, puis utilisés et mis en œuvre avec succès dans des missions de surveillance et d'enquête en situation réelle. Initialement, en associant des tests ELISA IgM anti-RVSV et ELISA de capture d'antigène, ainsi que le test RT-PCR, nous avons rapidement identifié le RVSV comme étant la cause d'un important foyer en Arabie Saoudite, en septembre 2000. Le test RT-PCR a complété les systèmes de détection ELISA antigène et anticorps pour le diagnostic rapide initial de la FVR. Cette épidémie de FVR en Arabie Saoudite, la première confirmée en dehors de l'Afrique, illustre le potentiel de propagation de cette maladie dans d'autres régions du monde. L'activité virale dans la péninsule arabique a entraîné beaucoup de cas de septembre 2000 à février 2001. Les résultats de notre recherche de six infections à virus FVR, confirmées en laboratoire chez des contacts familiaux confirment l'opinion selon laquelle les patients hospitalisés représentent une petite fraction du nombre de personnes infectées. Sur la base de ces observations et d'observations antérieures, le nombre d'infections humaines au cours de cette épidémie a dû être considérable.

La comparaison phylogénétique des différences de séquences nucléotidiques au niveau des segments S, M et L des souches FVR Péninsule Arabique et celles des souches FVR précédemment caractérisés a montré une relation étroite entre les virus FVR Arabie Saoudite et Yémen, avec ceux circulant auparavant en Afrique de l'Est, en particulier avec les souches incriminées lors de la grande épidémie dans la Corne de l'Afrique en 1997–98. Ces résultats sont cohérents avec l'hypothèse de l'introduction du virus de la fièvre de la FVR en Arabie Saoudite et au Yémen à partir de l'Afrique de l'Est.

Suite à cette détection initiale, le développement de la PCR en temps réel (RT-PCR) a permis de caractériser de façon plus poussée de cette épidémie, ainsi que de mettre en place une réponse pour étudier et caractériser les émergences futures. L'analyse de la séquence complète du génome de 40 souches de virus de la FVR biologiquement et écologiquement diverses a démontré que la diversité globale de nucléotides probablement résultant d'une origine commune récente. Ceci a fourni une base pour établir une technique rapide et à haut débit pour la détection en réel RT-PCR de l'ensemble de la diversité génomique connue du virus de la FVR.

L'utilité de ce test par rapport aux tests sérologiques standard a été validé à l'aide de sérums de patients humains prélevés lors de l'épidémie de FVR en Arabie saoudite en 2000. Comme prévu, la charge moyenne globale d'ARN du virus de la FVR chez les patients était significativement plus faible chez les patients qui ont survécu à l'infection, que chez ceux dont l'issue était fatale. Fait intéressant, même si la charge en ARN du virus de la FVR était initialement élevée parmi certains cas non mortels, nous avons constaté une diminution significative de la moyenne de la charge en ARN entre les jours 1 à 4, et 5 à 8 après l'apparition des symptômes. La combinaison de ces données avec les diminutions rapides de la charge d'ARN observée parmi les cas non mortels indique que les tests d'échantillons de patients en série recueillis 24 à 48 heures peuvent avoir une utilité pronostique pour déterminer la progression de l'infection chez les patients malades. Ces résultats mettent en évidence la nature complémentaire des tests de détection moléculaire et des tests sérologiques afin de caractériser les investigations épidémiologiques survenant dans des régions où le virus de la FVR est déjà endémique, c'est-à-dire avec un pourcentage d'individus qui a déjà été exposée au virus de la FVR.

À partir de 2011, la Viral Special Pathogens Branch (VSPB) du CDC, en collaboration avec l'UVRI et le Ministère de la Santé (MdS) Ougandais, a développé un programme national de surveillance et de laboratoire des FHV. Ce programme a permis la rapide identification de plusieurs foyers de FHV d'importance majeure. Le système de surveillance des FHV en Ouganda a contribué à une réduction spectaculaire du délai de réponse aux foyers de FHV. La détection précoce des cas et la confirmation en laboratoire a conduit à des réponses de santé publique plus rapide, à l'isolement des cas cliniques, à une diminution de la transmission secondaire dans les communautés, et finalement à des épidémies plus courtes et moins graves. Deux épidémies importantes d'Ebola-Soudan (SUDV) et d'Ebola Bundibugyo (BDBV) se sont produites en 2000 et 2007 respectivement, alors

que la surveillance et la capacité de détection des épidémies étaient limitées. Suite à la mise en œuvre de cette surveillance renforcée, le nombre de détections d'épidémies a été multiplié par cinq par rapport aux 10 années précédentes. La taille et l'étendue de chaque émergence ont également été considérablement réduite, le délai entre la notification clinique initiale et la confirmation en laboratoire étant raccourci de 2 semaines avant 2010 à une moyenne de seulement 2,5 jours. Cette réaction rapide et la maîtrise de ces épidémies témoignent de l'impact bénéfique considérable sur la santé publique d'une surveillance continue des FHV, diminuant donc leur morbidité et mortalité.

Soulignant les réussites du réseau de surveillance, les manuscrits sélectionnés décrivent la première ré-émergence de SUDV en 2011 après une absence de dix ans, et la première confirmation de la FVR en près de 50 ans. Ces deux épidémies décrivent le large éventail des réponses et du contrôle allant d'un cas isolé due à Ebolavirus à de multiples cas de FVR, comprenant des infections chez l'homme et le bétail. En plus de décrire la séroprévalence de la FVR en Ouganda et la caractérisation génétique des isolats viraux, la mise en place des enquêtes CAP a également permis de comprendre les croyances personnelles et pratiques qui contribuent à des comportements à risque élevé, ou identifier des professions à risque.

Ces multiples détections d'épidémies et leurs études ultérieures illustrent l'utilisation de techniques moléculaires développées en laboratoire et décrites dans cette thèse. Le programme de surveillance des FHV en Ouganda utilise à ce jour les tests de diagnostic moléculaire et sérologique présentés dans cette thèse, ce qui témoigne d'un profil de recherche large, des techniques de laboratoire, en passant par l'épidémiologie et une forte expérience de terrain. Parallèlement à ce travail de surveillance et d'épidémiologie en Ouganda, mes 20 ans d'expérience en santé publique et en recherche en laboratoire, témoignent d'un parcours professionnel diversifié et multidisciplinaire.

Cette expérience professionnelle m'a également permis de construire un vaste réseau de collaborateurs et de partenariats, scientifiques et en santé publique. Cela m'a amené plus particulièrement à développer une collaboration avec le CIRAD et l'université de Montpellier dans le but de mieux caractériser les maladies virales zoonotiques. Ceci passe par l'amélioration des méthodes de surveillance en santé humaine et animale, par la validation de nouvelles méthodes de diagnostic de laboratoire et par la caractérisation de facteurs entomologiques et environnementaux contribuant à l'émergence et la ré-émergence des FHV en Afrique. Ceci sera accompli conjointement grâce à l'expertise du CIRAD de l'Université de Montpellier, du Centers for Disease Control (CDC) et des partenaires gouvernementaux et non gouvernementaux en Ouganda.

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List of Acronyms

BAT	Buffered Brucella antigen test
BUGV	Bundibugyo ebolavirus
CFR	Case fatality rate
CIRAD	Centre de coopération internationale en recherche agronomique pour le développement
CCHF	Crimean-Congo hemorrhagic fever
CCHFV	Crimean-Congo hemorrhagic fever virus
Ct	Cycling threshold value
DNA	Deoxyribonucleic acid
EAVRI	East African Virus Research Institute
EBOV	Ebola virus
EVD	Ebola virus disease
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
GDP	Gross domestic product
HCIV	Health Center IV
HFV	Hemorrhagic fever viruses
HbSAg	Hepatitis B surface antigen
IDSR	Integrated Disease Surveillance and Response
KRRH	Kabale Regional Referral Hospital
KAP	Knowledge, attitudes, and practices
LOD	Limit of detection
MVD	Marburg virus disease
MARV	Marburgvirus
MCMS	Medical countermeasures
MAAIF	Ministry of Animal, Agriculture Industries and Fisheries
MOH	Ministry of Health
NSs	Nonstructural protein
NP	Nucleoprotein
OR	Odds ratio
PPE	Personal protective equipment
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RAVV	Raven virus
R&D	Research and Development
RESTV	Reston virus
RT-PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
SOPs	Standard operating procedures
SUDV	Sudan ebolavirus
SUMOD	Sum optical densities
TAFV	Tai forest virus
TBE	Tick-borne encephalitis
UAIS	Uganda AIDS Indicator Survey
UVRI	Uganda Virus Research Institute
VHFs	Viral hemorrhagic fevers
EPI	WHO Expanded Program on Immunization
YFRA	Yellow Fever Risk Assessment

Surveillance and epidemiology of viral hemorrhagic fevers (VHFs): Identification of emergence, seroprevalence, and risk factors of VHFs in Uganda

1 Introduction and Objectives:

Viral hemorrhagic fevers (VHFs) are a group of illnesses caused by several distinct families of viruses. The term viral hemorrhagic fever is used to describe a severe multisystem syndrome¹. While infection with some types of hemorrhagic fever viruses (HFV) results in relatively mild illness, many of these viruses cause severe, life-threatening disease in humans. Clinically, a VHF is characterized by acute onset of high fever with other accompanying non-specific signs and symptoms. These symptoms are often accompanied by hemorrhage (bleeding), but often not until the late stages of the disease. Typically, the overall vascular system is damaged following infection and the body's ability to self-regulate becomes impaired. Viruses in the genera Ebolavirus and Marburgvirus in the family Filoviridae are known to cause "classical" hemorrhagic fever disease. Other viral families that cause VHFs include Phenuiviridae (Rift Valley fever virus [RVFV]), Nairoviridae (Crimean-Congo Hemorrhagic Fever virus [CCHFV]), and Arenaviridae (Lassa virus).

Early clinical manifestations of VHFs include headaches, fever, malaise, anorexia, arthralgia, and varying degrees of nausea, vomiting, and diarrhea, which later progress into external or internal hemorrhages, renal failure, and shock. Patients infected with HFV exhibit these signs with varying degrees of severity and not all of them develop a classic hemorrhagic syndrome^{2,3}. During the early stages of an HFV infection, most patients present to a health care facility with non-specific symptoms that could also indicate other, more common tropical infections, such as malaria, typhoid, or rickettsial illnesses. Thus, properly identifying, isolating, and treating a patient with early-stage VHFs is often challenging.

All VHFs are zoonotic, with while some VHFs are transmitted by arthropod vectors such as CCHFV and RVFV, and can have a zoonotic, or animal reservoir host, such as livestock. Filoviruses like Ebola virus (EBOV) are transmitted by close contact with an infected person or their infected body fluids, or through direct or indirect contact with their natural reservoir host. Though the reservoir host for ebolaviruses has not yet been definitively identified, the Egyptian fruit bat, *Rousettus aegyptiacus*, has been shown to be the host of Marburgvirus (MARV)⁴. While viruses that cause VHFs are found worldwide, they are relatively restricted by the distribution of their reservoir host species.

Outbreaks of VHFs, most notably Ebola virus disease (EVD), Marburg virus disease (MVD), and Rift Valley fever (RVF), have been well documented across sub-Saharan Africa and cause widespread morbidity, mortality, and substantial socio-economic losses. There have been 27 confirmed outbreaks of EVD reported since its first identification in 1976 in Zaire (now the Democratic Republic of Congo [DRC]), and 13 outbreaks of MVD since its first identification in Germany in 1967¹. The largest of these outbreaks by far has been the West African EBOV outbreak in 2013–2016,

followed by the EBOV outbreak in DRC, which is ongoing at the writing of this manuscript, and the outbreak of Sudan ebolavirus (SUDV) in Uganda in 2000. The economic impacts of the West African EVD outbreak alone are estimated at over \$2.2 billion (US) in GDP from loss of investments, significant drops in private sector growth, lower food and agricultural production, and decreased trade⁵. The costs of the response to the outbreak by the affected countries and the international community exceeded \$3.6B.

RVFV has also caused widespread outbreaks in human populations across Africa. The most significant socio-economic losses are due to morbidity and mortality of affected livestock. Because of the ecological, zoonotic, and vector-borne nature of RVFV, outbreaks tend to primarily affect regions with large livestock populations and countries in which the local economy is highly dependent on livestock and agricultural trade. Of the major RVF outbreaks recorded since 1930, socio-economic impact has ranged from \$7M to over \$470M⁶, accounting for losses of over 5% of GDP. Other costs associated with RVF outbreaks are due to impact on local and international trade and public health and healthcare expenditures.

Due to the continuing threat and increased potential of VHFs like EVD, MVD, and RVF to cause such significant negative impact on the health, well-being, and economies of affected countries, WHO initiated the Research and Development (R&D) Blueprint for these and other priority pathogens⁷. The R&D Blueprint is a global strategy and preparedness plan that provides a framework for prioritizing research areas and activities to accelerate the collaborative development of medical countermeasures, including diagnostics, therapeutics, and vaccines against these priority pathogens. Fast-tracking the development and delivery of these countermeasures will save lives and avert future large-scale epidemics.

Inclusion of priority VHFs into the WHO R&D Blueprint underscores their importance and threat to global public health. To develop more successful countermeasures for effective control and containment of VHF outbreaks, the epidemiology and underlying risk factors of these pathogens must be understood, along with the molecular and clinical characteristics that contribute to their emergence. Although much progress and data have already been contributed to this R&D blueprint, many key gaps in knowledge remain for some of the priority pathogens, including EBOV and RVFV. A better, more comprehensive understanding of the ecology, epidemiology, pathogenicity, and genomic factors of these and other viruses will increase understanding of their potential for emergence. This would improve human and zoonotic surveillance, allow more sensitive and specific diagnostic testing, and characterize the incidence and prevalence of these viruses in endemic areas to implement control and prevention strategies. The primary aim of this dissertation is to study scientific and epidemiological characteristics of VHFs, using EBOV and RVFV as primary examples, with the goal of contributing knowledge that will allow for improved surveillance, early detection, investigation, control, and prevention of large VHF outbreaks. This specific aim was accomplished by carrying out the following series of objectives:

1. Use existing molecular and serological techniques to identify and genetically characterize the first RVFV outbreak identified outside of the African continent⁸.
2. Develop and validate a new sensitive and broadly reactive quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) assay for rapid laboratory detection of RVFV⁹.
3. Design and implement a comprehensive VHF surveillance system in Uganda to rapidly detect, respond to, and control VHF outbreaks¹⁰.
4. Describe the effectiveness of the Uganda VHF surveillance system and quantify key response and control indicators¹¹.
5. Genetically compare filovirus isolates obtained from Uganda VHF surveillance work and analyze these genomes for potential genetic virulence factors contributing to continued pathogen re-emergence¹².
6. Utilize multiple investigations and studies to characterize the re-emergence of RVFV in Uganda:
 - a) Perform a comprehensive epidemiological investigation and phylogenetic analysis of the RVFV isolates to identify the source(s) of the outbreak¹³.
 - b) Determine the seroprevalence of RVFV infection in humans and domestic livestock species to identify risk factors for RVF in select areas in Uganda¹⁴.
 - c) Describe the knowledge, attitudes, and practices associated with RVF in Uganda¹⁵.

The chart presented in Figure 1 below shows how the selected manuscripts included in this thesis are related, each building off the knowledge from previous manuscripts to cohesively connect the objectives listed above.

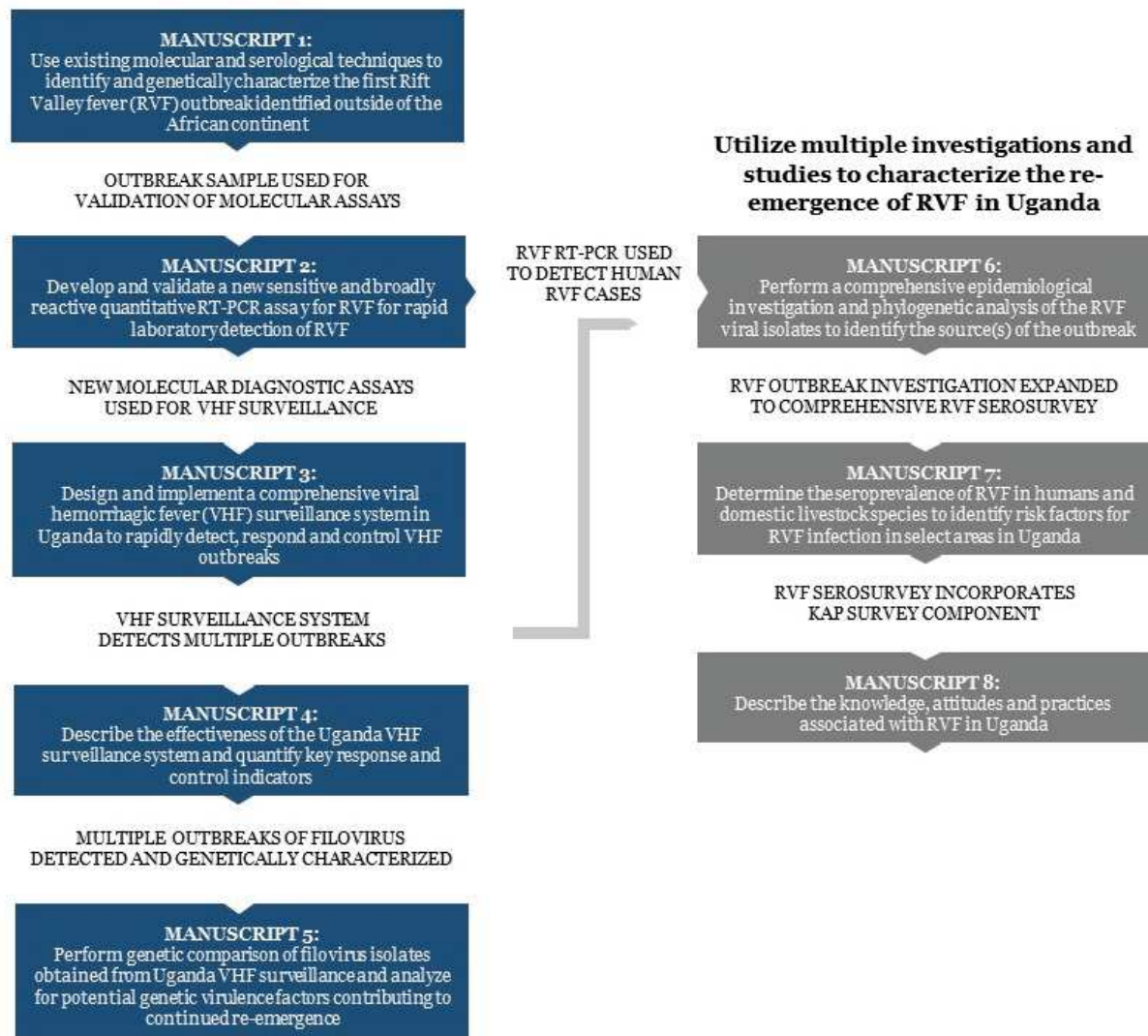


Figure 1: Chart representing the selected manuscripts and the corresponding objective, showing the linkages between them and demonstrating the contribution of each to the body of the thesis.

2 Background

2.1 Epidemiology, ecology, and clinical presentation of VHFs

2.1.1 Ebolaviruses

EVD is a severe illness associated with hemorrhage and high fatality rates, and is thought to be of zoonotic origin. EVD is caused by 5 species in the genus *Ebolavirus* in the family *Filoviridae*: *Zaire ebolavirus* (more commonly known as ebolavirus or EBOV), *Sudan ebolavirus* (SUDV), *Tai Forest ebolavirus* (TAFV), *Bundibugyo ebolavirus* (BDBV), and *Reston ebolavirus* (RESV). A sixth species, *Bombali ebolavirus*, has recently been identified in bats in Sierra Leone, but

has not been associated with human infection¹⁶. These ebolavirus species have been identified in outbreaks from geographically diverse parts of sub-Saharan Africa and epidemiologically have shown differing pathogenicity¹⁷ (Figure 2). EBOV was first described in 1976 in what is now DRC¹⁸, and was responsible for the largest outbreak on record in West Africa in 2013–16¹⁹ as well as the ongoing, second-largest outbreak in DRC. SUDV has been detected only in South Sudan and Uganda, whereas BDBV has been reported in both Western Uganda and Isiro district in the neighboring eastern DRC^{20, 21}. TAFV was reported in a single non-fatal case in West Africa in 1994²². No fatal human cases have been associated with RESV, which was first isolated in the USA from monkeys imported from the Philippines²³.

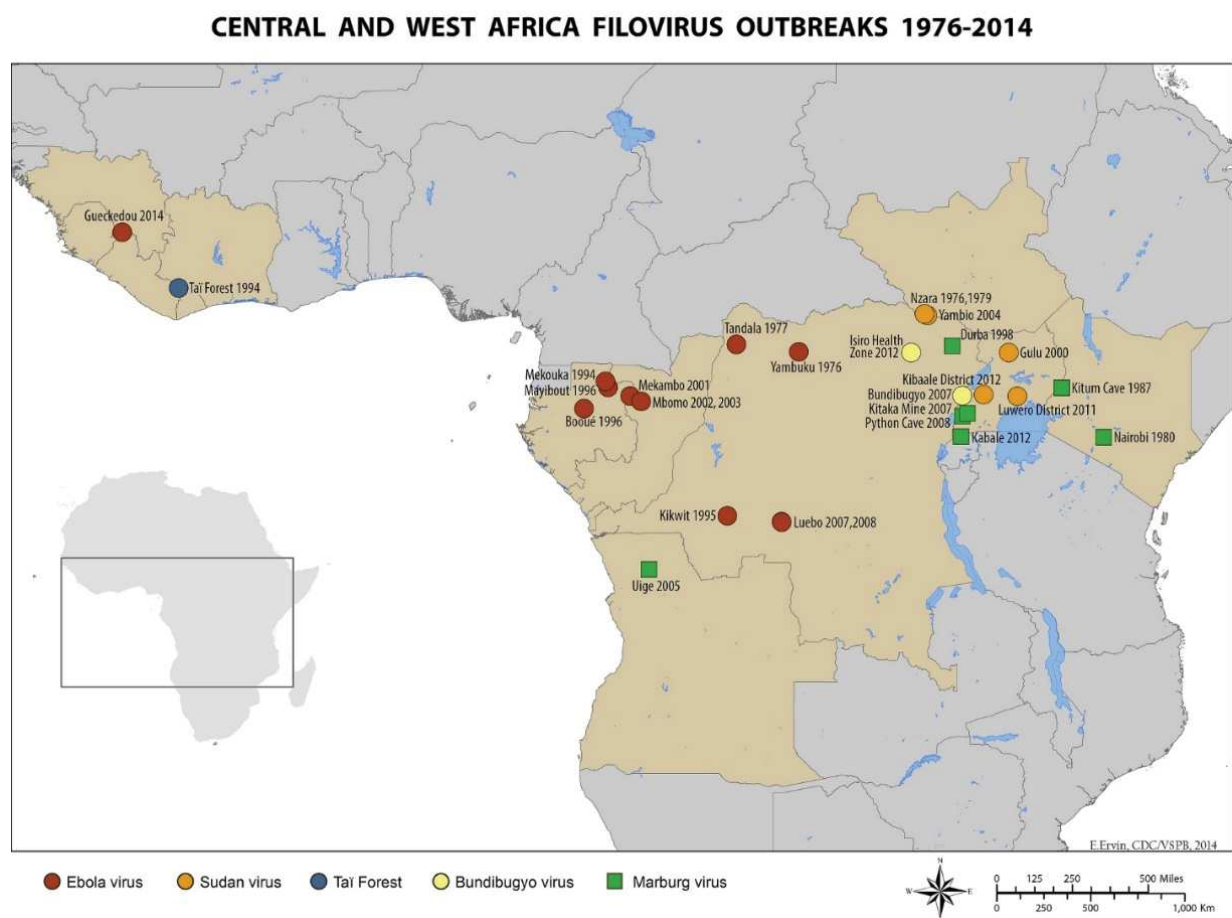


Figure 2: Map representing filovirus outbreaks across sub-Saharan Africa from 1976–2014¹

The early stages of EVD typically manifest as fever, headaches, and myalgia, followed by gastrointestinal symptoms such as diarrhea, vomiting, abdominal pain, and dehydration. If not identified early and properly treated, the infection can progress to a hemorrhagic phase with bleeding from multiple orifices, produce neurological symptoms, and lead to shock that is often fatal. In the West African EVD outbreak, the clinical signs reported most frequently were fever (87.1%), fatigue (76.4%), loss of appetite (64.5%), vomiting (67.6%), diarrhea (65.6%), headache (53.4%), and abdominal pain (44.3%). Specific hemorrhagic symptoms were rare, with bleeding of unexplained origin reported in only 18.0% of cases²⁴. During a SUDV outbreak in northern Uganda in 2000, all laboratory-confirmed cases presented

as febrile, and other frequently documented symptoms were asthenia, loss of appetite, cough, nausea or vomiting, and diarrhea²⁵. Specific symptoms do not usually present during the incubation period, which ranges between 2–21 days. Identifying suspected EVD cases depends on the clinical symptoms and epidemiological links to confirmed or probable cases.

Ebolaviruses are thought to transmit from wildlife to the human populations through direct or indirect exposure to infected bat species (Figure 3). Bats have long been the suspected reservoir of ebolaviruses, and ebolavirus RNA and anti-ebolavirus antibodies have detected in some bat species^{16, 26}. However, until the recent identification of *Bomboli ebolavirus*, no ebolavirus has been directly detected or isolated from bats. Non-human primates have long been recognized as potential sources of human ebolavirus infection, but their role as potential reservoirs is not widely accepted, since they are as susceptible to ebolavirus infection as humans²⁷⁻³⁰.

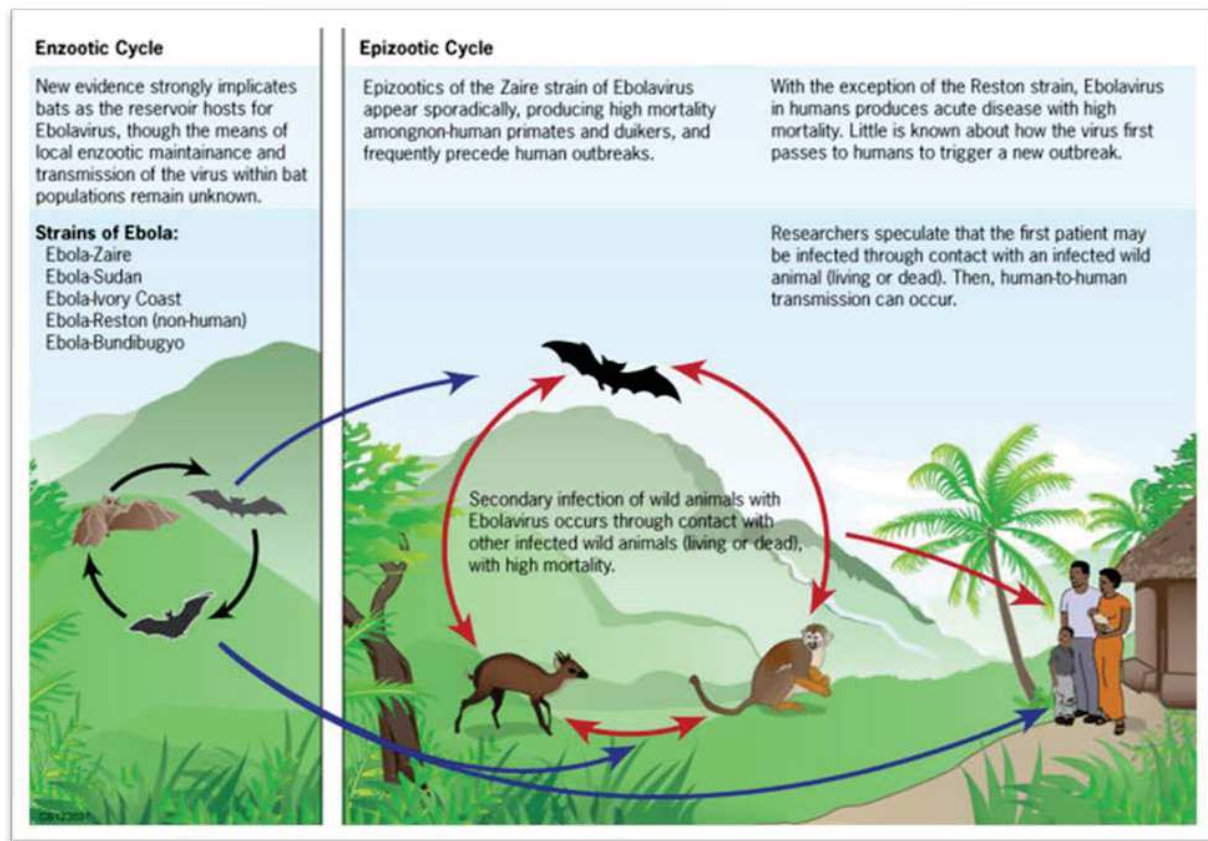


Figure 3: Ebolavirus ecology¹

2.1.2 Marburgvirus

MVD is caused by MARV, which, like ebolaviruses, is also included in the filovirus family. MVD has similar characteristics to EVD, and is a severe, potentially fatal illness in humans and non-human primates, characterized by hemorrhagic signs indistinguishable from EVD. The disease was first described in 1967 in the German city of Marburg,

where non-human primates imported from Uganda infected laboratory and animal care workers³¹. The incubation period of MARV ranges from 2 to 21 days, and early symptoms of MVD include sudden fever, fatigue, headache, nausea and vomiting, diarrhea, rash, and conjunctivitis. As the disease progresses, hemorrhagic signs are also accompanied by failure of multiple organs and disseminated intravascular coagulation. During a multidistrict MVD outbreak in 2012 in Uganda, nearly all confirmed and probable cases (96%) had fever, anorexia, fatigue, headache, and vomiting³², and half of confirmed and probable patients (50%) showed hemorrhagic symptoms. MVD, like other related VHF, first manifests with non-specific symptoms, and definitive diagnosis relies on laboratory confirmation³³.

In 2007, the CDC Viral Special Pathogens Branch (VSPB) viral ecology team identified *R. aegyptiacus* bats as the reservoir for MARV^{4, 34} (Figure 4). The identification was suggested after 2 tourists were retrospectively identified as having been infected with MARV after visiting a cave located in Queen Elizabeth National Park in Uganda; this cave was populated with a large colony of *R. aegyptiacus* bats. Additionally, a group of miners became infected with MARV after working in a nearby artisanal mine, which also hosted a large *R. aegyptiacus* fruit bat colony²⁶.

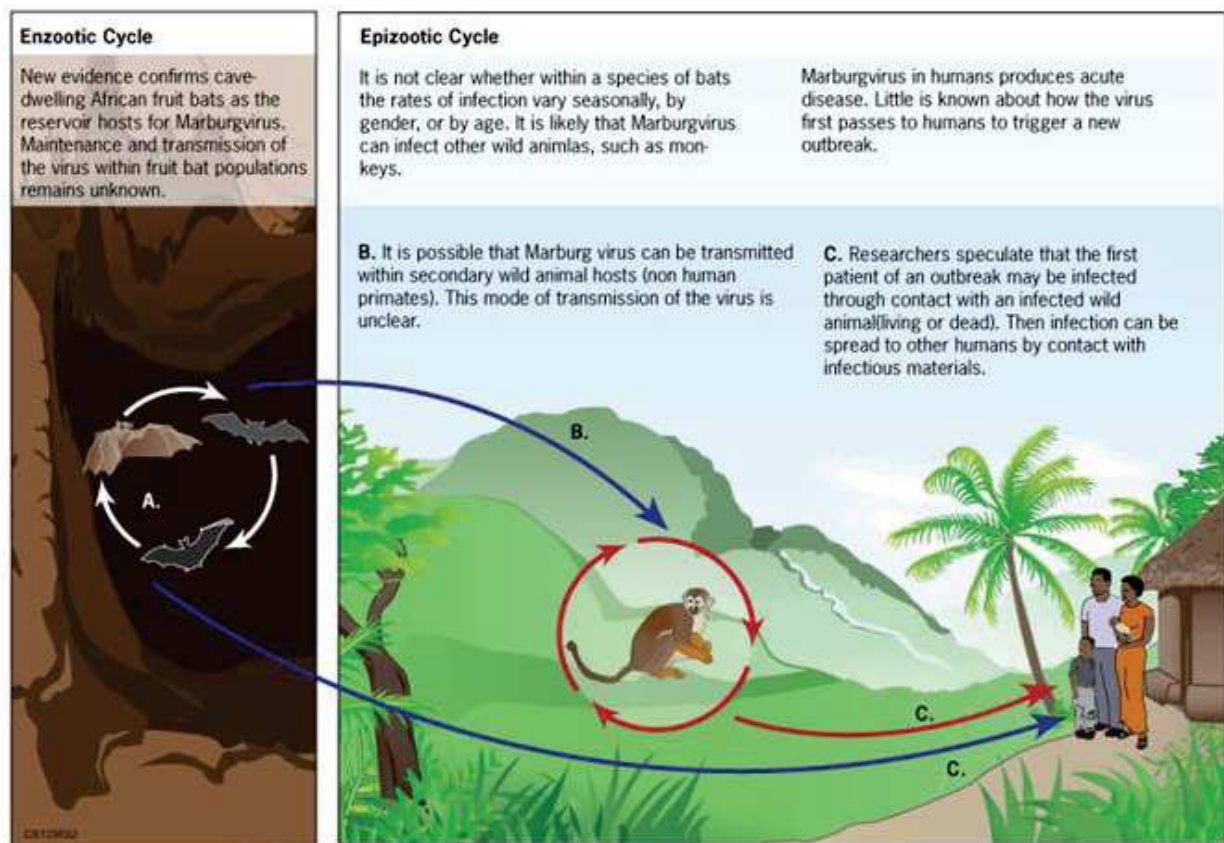


Figure 4: MARV ecology¹

2.1.3 Crimean-Congo hemorrhagic fever virus

Crimean-Congo hemorrhagic fever (CCHF) is an ancient viral disease, with human cases probably occurring as far back as the 12th century in present-day Tajikistan and other parts of central Asia. CCHFV was formally recognized in 1944 during an outbreak in the Crimean Peninsula^{35, 36}. After the causative agent of this outbreak was characterized, it was later found to be related to a virus isolated from a febrile patient in the Kisangani area of the DRC in 1956^{37, 38}, leading to the combined name Crimean-Congo hemorrhagic fever virus. CCHFV is the most widely geographically distributed tick-borne disease in the world, affecting both animals and humans mainly in Eastern Europe, Asia, Middle East, and Africa³⁹ (Figure 5). In animals, infection is mainly asymptomatic, while in humans, it can manifest as an acute and highly infectious VHF that readily transmits to close contacts, with a case fatality rate of over 50% in hospitalized patients^{40, 41}.

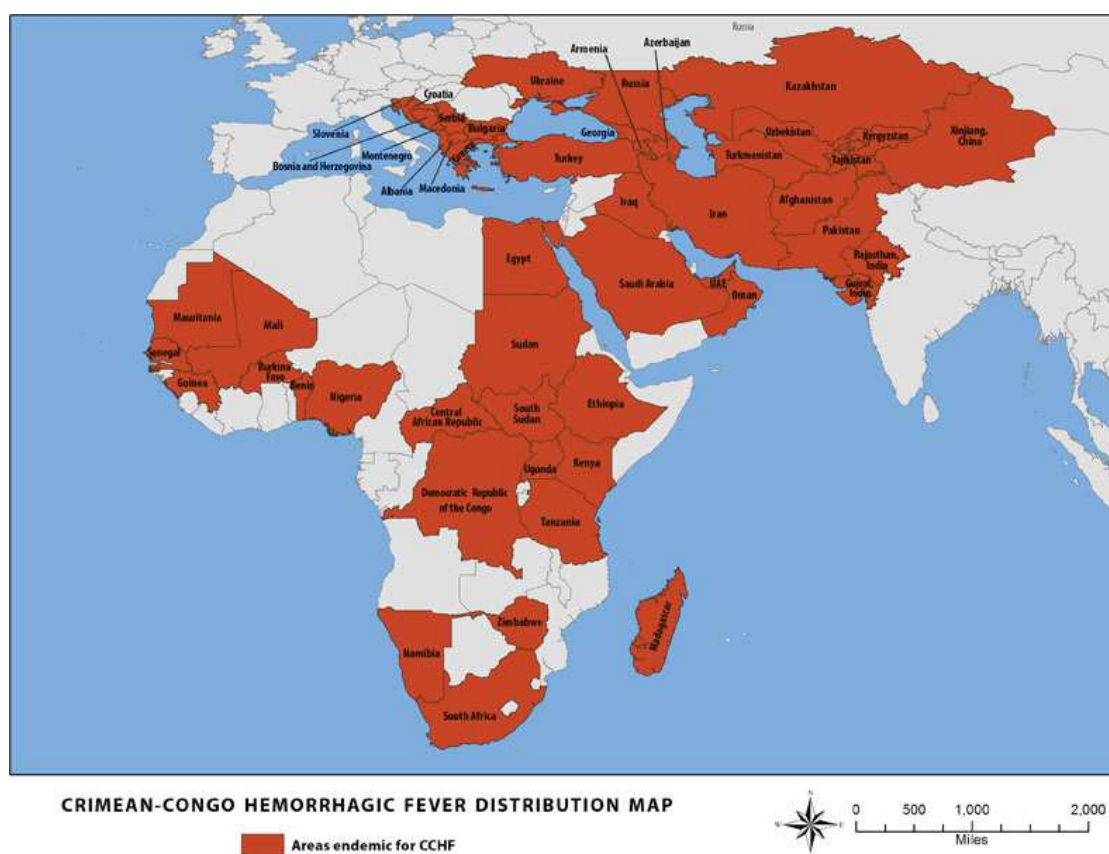


Figure 5: CCHFV distribution map.¹ (as of 2014)

CCHFV is an enveloped, negative-sense RNA virus in the *Bunyvirales* order, family *Nairoviridae*, and genus *Orthonairovirus*. It is maintained in nature by tick vectors, principally *Hyalomma* species, through tick-to-tick enzootic and vertebrate-mediated epizootic cycles (Figure 6). Virus spillovers to humans usually occur through tick bites and direct contact with viremic animals and other infectious biological materials^{42, 43}. Persons most at risk for CCHFV

infection include individuals working in agricultural fields and abattoirs, and herdsmen who may be exposed to ticks as well as infectious animal products like milk and meat. Secondary transmissions are also common among medical staff, other hospital patients, and close contact family members and relatives who are exposed through infectious blood or other body fluids of acutely sick patients.

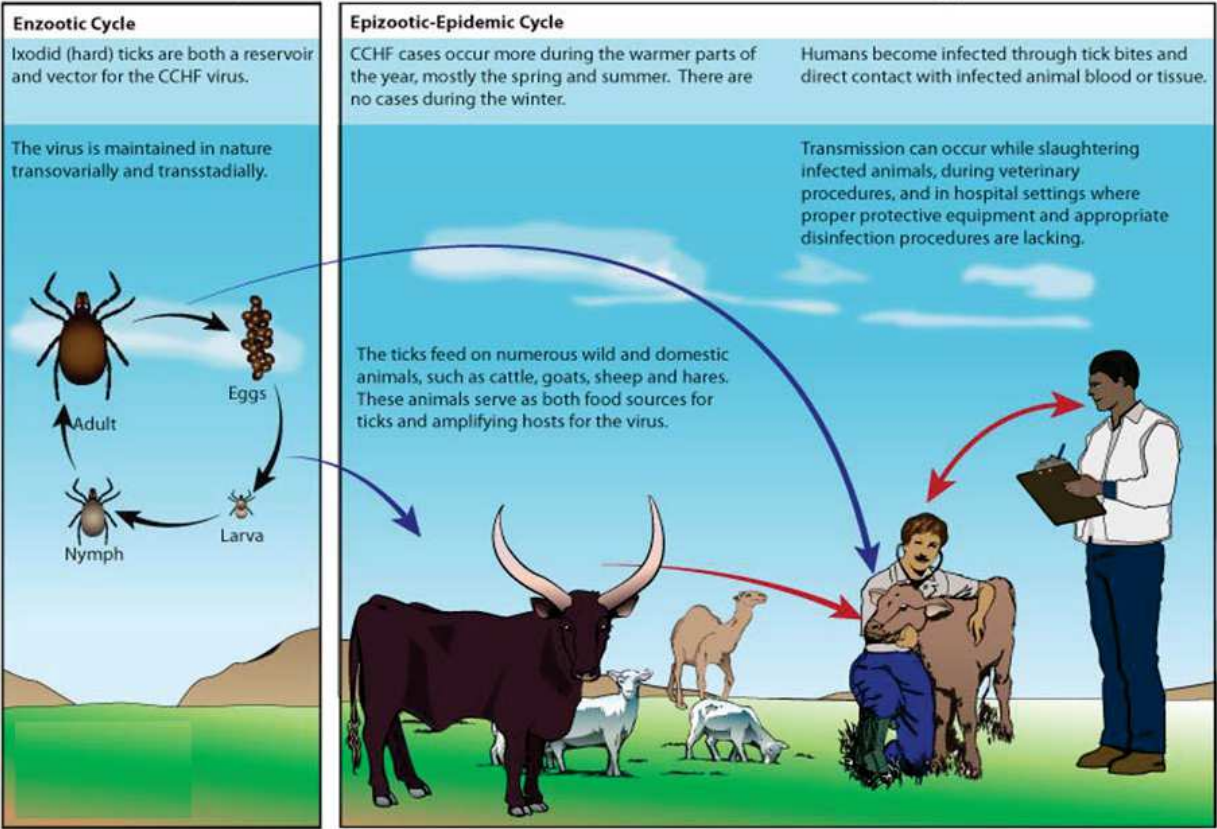


Figure 6: CCHFV ecology¹

Whereas CCHF is well studied in Europe and now increasingly in Asia⁴⁴, its epidemiology in sub-Saharan Africa is much less defined⁴⁵, with most available country information dependent on serological and vector presence^{46, 47}. Given its widely believed potential to cause a future large public health emergency, it was recently listed by WHO as a top priority emerging disease requiring accelerated efforts in surveillance, research, and diagnostics development⁴⁸.

2.1.4 Rift Valley fever virus

2.1.4.1 Epidemiology and ecology of RVF

RVF is caused by RVFV, a virus in the *Bunyavirales* order, family *Phenuiviridae*, genus *Phlebovirus*. It is an emerging epidemic disease of humans and livestock, and an important endemic problem in sub-Saharan Africa⁴⁹. RVFV is transmitted to livestock and humans by the bite of infected mosquitoes or exposure to tissues or blood of infected

animals⁵⁰. Interepidemic virus maintenance is thought to occur either transovarially in *Aedes* species mosquitoes, or through cycling of low-level transmission between mosquitoes and domestic livestock or wild ungulates⁵¹. After periods of heavy rainfall, *Aedes* mosquitoes rapidly emerge, resulting in extensive amplification of the virus through infection of livestock⁵⁰.

Clinical presentation of RVF in animals varies depending on the species and ranges in severity. Livestock, particularly cattle, sheep, and goats, are highly susceptible to RVFV and present with symptoms of fever, loss of appetite, weakness, low milk production, nasal discharge, vomiting, and diarrhea. During large epizootics, “abortion storms,” particularly in sheep and cattle, have been recorded. High newborn mortality (80–100%) and adult mortality (5–20%) may also occur⁵¹.

Humans infected with RVFV can present with varying degrees of symptomatology ranging from asymptomatic or mild, self-limited febrile illness, to with severe jaundice, rhinitis, encephalitis, and hemorrhagic manifestations in a small number of cases (<8%). Retinal degeneration (5–10% of cases), hemorrhagic fever (<1%), or encephalitis (<1%) may also develop^{8, 52 53}. Laboratory findings in patients with RVF can include leukopenia, thrombocytopenia, and elevated liver enzymes. About 1% of RVF cases progress to hemorrhagic disease. The overall fatality rate is estimated to be 0.5–1%, but in the 2006–2007 outbreak in Kenya, the case fatality rate was reported to be as high as 29%⁵⁴.

Although low-level RVFV activity most likely occurs throughout enzootic regions each year, the emergence of RVFV in large epidemic and/or epizootic cycles is typically associated with unusually heavy rainfall and the emergence of the natural reservoir host, which is thought to be primarily transovarially infected *Aedes* spp. floodwater mosquitoes⁵⁵ (Figure 7). During large epidemics⁵⁶ and epizootics, the high numbers of infected individuals can greatly strain the capacity of the public health and veterinary infrastructure to provide rapid real-time diagnostic testing and basic medical care for infected individuals or animals.

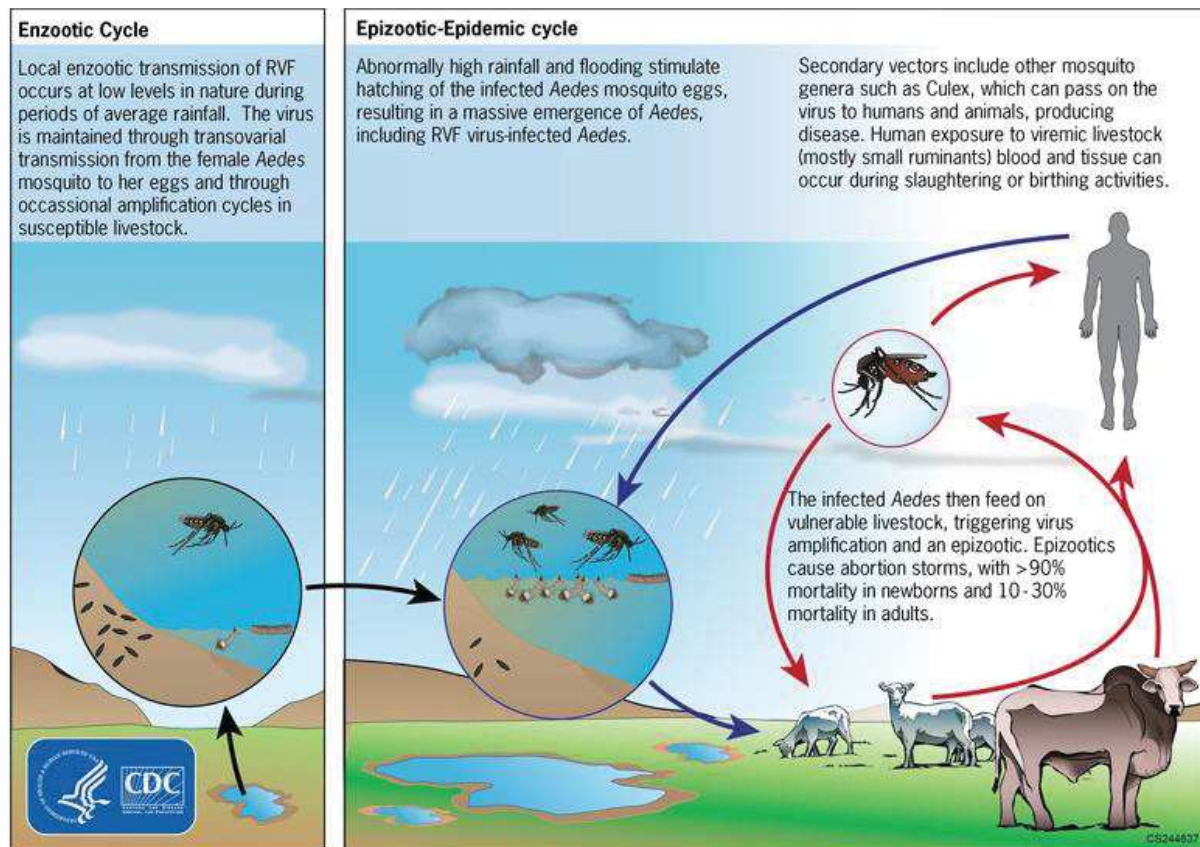
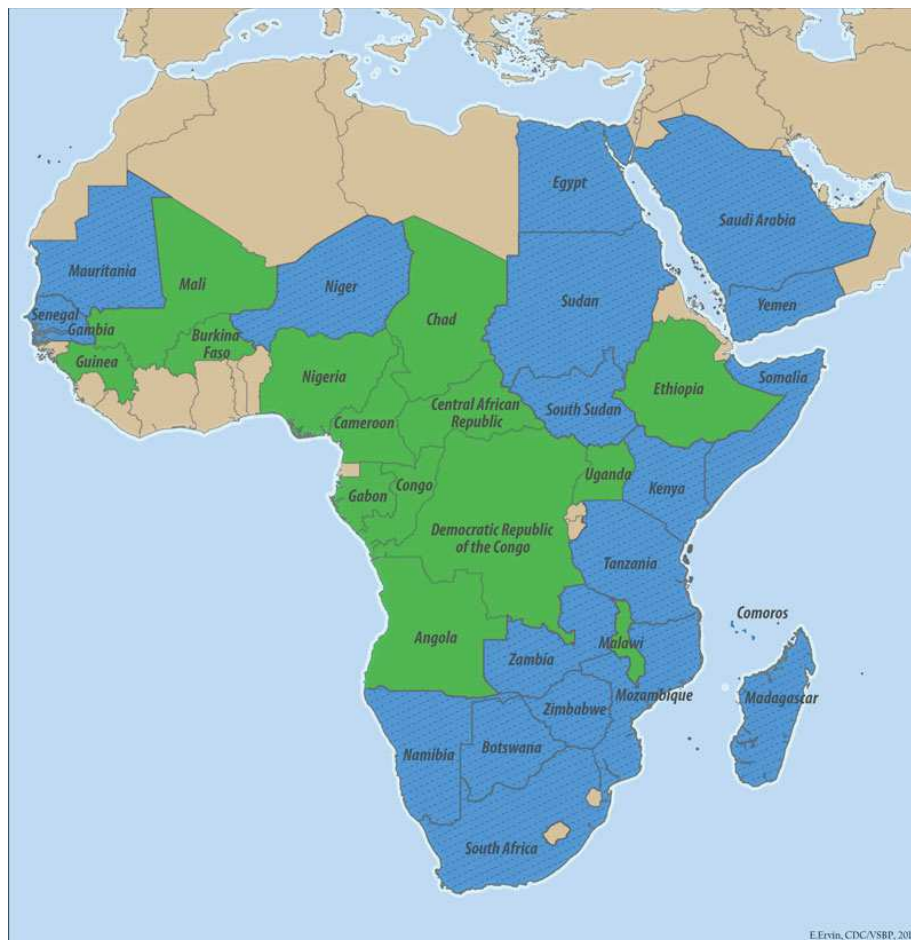


Figure 7: RVFV ecology¹

The ability of RVFV to cross international and natural boundaries is well documented. In 1977, it was first recorded north of the Sahara Desert in Egypt, and resulted in a massive epizootic and epidemic in that country, during which more than 200,000 people were estimated to have been infected. In 1979, RVFV was identified for the first time outside of continental Africa on the island of Madagascar⁵⁷. Later, in 2000, the virus was isolated for the first time outside of Africa across the Red Sea in Saudi Arabia and Yemen⁵⁸ (Figure 8). The potential of further introductions of RVFV into previously unaffected countries via infected livestock importation, mosquito translocation, human travel, or intentional release highlights the need for continued surveillance, prevention, and intervention measures.



Rift Valley Fever Distribution Map

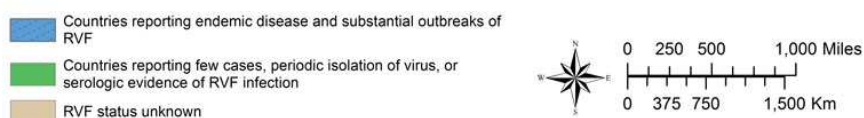


Figure 8: RVFV distribution map.¹ (as of 2016)

Currently, no RVFV vaccine is approved for use in either humans or animals in North America or Europe⁵¹. Some inactivated and live-attenuated vaccines have been developed, however, and shown to be efficacious in animals^{59, 60}. Development of human RVFV vaccines has been challenging due to vaccine safety. To better understand the utility of RVFV vaccines in a particular setting, the prevalence of disease in humans and animals must first be understood.

In agricultural communities, RVF outbreaks can cause significant economic losses⁵¹. The 2007 RVF outbreak in Kenya impacted agricultural production and employment, resulting in an estimated loss of \$32 million USD, due to both direct effects from livestock deaths and indirect effects that included reduced income from the closure of livestock markets and reduced sales of animal-derived products⁶¹. Outbreaks typically occur after periods of heavy rainfall and

flooding lead to increased mosquito populations. Indeed, in 1997–1998, large RVF outbreaks in northeastern Kenya occurred following El Niño rains and floods, resulting in many deaths in livestock and humans^{62, 63}. Infection in humans was tightly associated with contact with livestock and animal body fluids. RVF outbreaks were not reported again in East Africa until 2006–2007, when large numbers of humans and livestock were infected in Kenya, Somalia, Tanzania, and Sudan^{64, 65}. Studies following these outbreaks reported that herdsman and other individuals who handle or consume products from infected animals were at highest risk of infection. Additionally, outbreaks in East African countries mainly occurred in areas with poor soil drainage and flat lowlands that are less than 500 m above sea level, confirming the relationship between flood conditions and outbreaks^{66, 67}. Likewise, RVF outbreaks in humans and animals following flooding have occurred in Sudan⁶⁸, Saudi Arabia⁶⁹, Yemen (2000–2001), South Africa, and Egypt⁷⁰. Knowing that the risk of large RVFV epidemics significantly increases during periods of flooding and that disease occurs largely in herders, it is possible to use geospatial analysis to predict areas with increased risk of RVF outbreaks^{55, 71, 72}. Early identification of cases in livestock and humans is an important tool in outbreak detection and may aid in the rapid deployment of disease control measures, such as animal vaccines or mosquito control. Since RVFV vaccines have not yet been approved for human use, the main methods of disease prevention in people are early detection, awareness, and behavior modification by high-risk groups, such as using personal protective equipment (PPE) and increased hand hygiene. Therefore, it is important to ensure that at-risk populations in endemic regions are aware of RVF and of measures that can prevent infection.

2.1.4.2 Genetic analysis of RVFV in Africa and the Arabian Peninsula

RVFV is an enveloped, single-stranded, negative-sense RNA virus consisting of 3 RNA segments varying in size: large (L), medium (M), and small (S). The tripartite, negative-sense, single-stranded RNA genome of RVFV virus contains the small ambisense segment (S segment) encoding the nucleoprotein and the nonstructural proteins, the medium segment (M segment) encoding the polyglycoprotein precursors, and the large segment (L segment) containing the virus RNA-dependent RNA polymerase⁷³. The S segment encodes the nucleoprotein (NP) in the antigenomic sense and the nonstructural (NSs) protein in the genomic sense⁷⁴. The M segment encodes several viral proteins in a single open reading frame, including the 14 kDa NSm, 2 major envelope surface glycoproteins (Gn and Gc), and a 78 kDa fusion protein of NSm and Gn. The NSm proteins are non-essential for in vitro virus growth⁷⁵, but contribute to virulence in the mammalian host⁷⁶.

The first confirmed RVF outbreak outside Africa was reported in September 2000 in the Arabian Peninsula. By February 2001, a total of 884 hospitalized patients were identified in Saudi Arabia, with 124 deaths. In Yemen, 1,087 cases were estimated to have occurred, with 121 deaths⁶⁹. Laboratory diagnosis of RVFV infections included genetic detection of the virus and characterization of clinical specimens by RT-PCR, in addition to serological tests and virus isolation. Genetic analysis of selected regions of RVFV S, M, and L RNA genome segments indicated little genetic variation among the viruses associated with disease. The Saudi Arabia and Yemen RVFV isolates were almost identical

to those associated with earlier RVF epidemics in East Africa, indicating that genetic re-assortment did not play an important role in the emergence of this virus on the Arabian Peninsula. The genetic analysis of RVFV identified in Saudi Arabia and Yemen will be described in further detail below in Objective 1.

In late autumn of 2006, following unusually heavy rainfall, reports of human and animal illness consistent with RVF emerged across semiarid regions of the Garissa District of northeastern Kenya and southern Somalia⁷⁴. A total of 3,250 specimens from a variety of animal species, including domesticated livestock (cattle, sheep, goats, and camels) and wildlife, collected from a total of 55 of 71 Kenyan administrative districts, were tested by molecular and serologic assays. Evidence of RVFV infection was found in 9.2% of animals tested and across 23 districts, reflecting the large number of affected livestock and the geographic extent of the outbreak. The complete S, M, and/or L genome segment sequences were obtained from a total of 33 wild-type virus isolates collected from throughout Africa and Saudi Arabia between 1944 and 2000. Extensive genomic analyses demonstrated the concurrent circulation of multiple virus lineages, gene segment re-assortment, and the common ancestry of the 2006–2007 outbreak viruses with those from the 1997–1998 East African RVF outbreak. Complete genome sequencing of multiple isolates of RVFV revealed that the overall virus genomic diversity is low (~5%) at the nucleotide level⁷⁷. These findings impacted further studies of basic RVFV ecology and the design of broad-based surveillance and diagnostic methods to detect a diverse array of RVFVs, and will be described in further detail below in Objective 2.

2.2 VHF surveillance system in Uganda

Uganda has reported more VHF outbreaks than any other country in Sub-Saharan Africa, including 5 EVD outbreaks, 4 MVD outbreaks, and multiple outbreaks of CCHF and RVF. The first EVD outbreak in Uganda (which has remained the most significant EVD outbreak ever recorded in that country) occurred in 2000 in the districts of Gulu, Masindi, and Mbarara and involved 425 cases with 224 deaths (CFR 53%)⁷⁸. Since then, 4 additional EVD outbreaks have occurred in Uganda, including in Bundibugyo District in 2007 (147 cases, 37 deaths)²⁰, in Luweero District in 2011 (1 case, 1 death)¹⁰ and in 2012 (7 cases, 4 deaths)¹², and in Kibaale District in 2012 (24 cases, 17 deaths). The outbreak in Bundibugyo District was attributed to a new strain of Ebolavirus, later named BDBV⁷⁹; this strain subsequently caused an outbreak in Isiro, DRC, in 2012 (72 cases, 31 deaths²¹).

Four MVD outbreaks have been recorded in Uganda. The first occurred in 2007, and involved 3 cases and 1 death⁸⁰. In a 2012 outbreak, the total count of confirmed and probable MVD cases was 26, of which 15 (58%) were fatal³². The outbreak in 2012 started in Ibanda District and subsequently spread to at least 3 other districts. In 2014, Uganda reported a single MVD case from Mpigi District, and in 2017, 4 confirmed cases of MVD were identified in Kween District in eastern Uganda.

In addition to EVD and MVD, since 2013, multiple sporadic outbreaks of CCHF and RVF have been and continue to be detected and confirmed in multiple districts across Uganda.

The purpose of any effective public health surveillance system is to enable the prompt recognition of and inform rapid and appropriate responses to disease outbreaks. Important trends or new potential disease threats must be quickly recognized. Additionally, hypotheses regarding causes of re-emergence or changing patterns of the hemorrhagic diseases and allocation of resources for continued surveillance and research into VHFs can lead to improved planning, development, and policies for mitigating VHF impact. Thus, development and maintenance of routine and enhanced VHF surveillance is critical, especially in Uganda, to detect, confirm, and rapidly respond to any possible threat of VHF.

Uganda's VHF surveillance system is a highly functional epidemiological and laboratory surveillance system involving health facilities from across the country and a diagnostic laboratory that provides timely results to the health facilities that submit samples from suspect cases. The primary objectives of the VHF surveillance program were to:

- Provide direct support to enhance Uganda's capability to detect, diagnose, and report VHFs and other emerging zoonotic infectious diseases.
- Improve Uganda's capability to respond and control outbreaks of VHF outbreaks.
- Conduct ongoing viral ecology research for filoviruses and other VHFs in Uganda.
- Collaborate with other CDC partners for disease surveillance and epidemiological and ecological studies.
- Coordinate with Global Health Security program in Uganda since 2013 for improved surveillance coordination, case identification, reporting, and sample transport from districts throughout Uganda.
- Ensure the sustainability of all provided and developed capabilities and capacities within Uganda's current priorities and budget.

This comprehensive program was implemented through the creation of a series of sentinel surveillance sites directly linked to the Uganda Virus Research Institute (UVRI) and the Ugandan Ministry of Health (MOH). Regional hospital and district health office staff were trained in epidemiological data collection, VHF surveillance, epidemiology and ecology, suspect patient identification, and infection control procedures (Figure 9). Key elements of the program were development of a standardized 3-tiered case definition and case reporting form specific for VHFs. Each site can report a suspect case and then safely collect and ship samples to be tested at UVRI and reported to MOH. The program initially started with 6 directly supported sentinel surveillance sites and has now grown to over 20 sites.

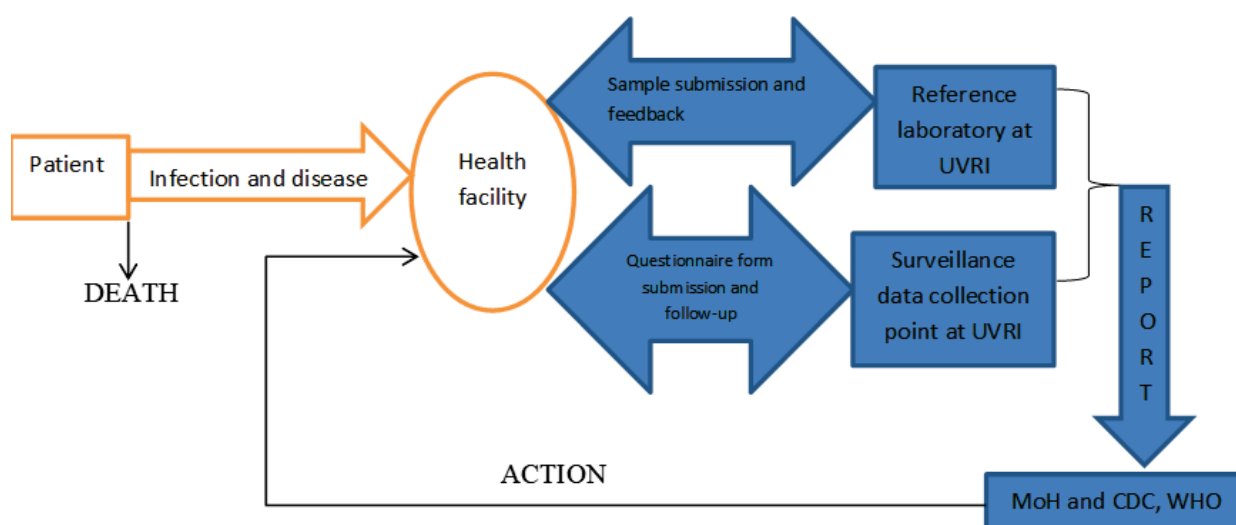


Figure 9: Schematic for implementation of the Uganda VHF national surveillance system

2.3 RVFV Emergence in Uganda

RVFV in Uganda was first detected in mosquitos collected in Semliki Forest, Western Uganda, in 1944,⁸¹ and has since been detected several times by the East African Virus Research Institute (EAVRI; now UVRI). Human cases were recorded during outbreaks occurring near Entebbe in 1960 and 1962.^{82, 83} Until 2016, no large RVF outbreaks have been reported there since 1968, when 7 human cases occurred near Entebbe⁸⁴. Since then, serological evidence of human and livestock RVFV infections in Uganda has been intermittently reported^{85, 86}. A 2013 serological survey of goats in Ssembabule, Mpigi, Masaka, and Mubende Districts in Uganda showed a seroprevalence of 9.8%, suggesting RVFV circulation⁸⁷.

On March 10, 2016, UVRI and the Uganda MOH received a report of a VHF case presenting to Kabale Regional Referral Hospital (KRRH) in Kabale District in southwestern Uganda. The initial patient was a 48-year-old male butcher who had been working in a local abattoir. The patient presented with a history of fever, vomiting, diarrhea, headache, and hemorrhagic symptoms (bleeding gums, epistaxis, bloody urine and stools). A blood sample was collected and sent to the UVRI VHF laboratory for testing. On March 11, a second suspected VHF patient presented to KRRH with similar symptoms; a blood sample was collected for testing. This patient was a 16-year-old male student first reported by Kabale District Health Office from the Uganda/Rwanda border village of Katuna. Both samples were tested for hemorrhagic fever viruses, including ebolaviruses, MARV, CCHFV, and RVFV. RT-PCR and IgM serology showed both patients to be positive for RVFV. In total, 4 cases of acute, non-fatal human disease were identified during this outbreak, 3 by RVFV-specific RT-PCR and 1 by IgM and IgG serology.

The UVRI in Entebbe has been implementing laboratory-based VHF surveillance in Uganda since 2010, including testing for RVFV⁸⁸. The 2016 RVFV outbreak represented the 10th independent VHF outbreak detected and confirmed through this program, and the first time RVFV was detected in Uganda in 48 years. Because not all human or animal RVF cases are symptomatic, RVFV infections are often undetected. Thus, UVRI, the Ugandan MOH, Ugandan Ministry of Agriculture Animal Industry and Fisheries (MAIF), and the CDC collaborated on a study to assess the seroprevalence of RVFV in humans and animals living in and around Kabale District. The objectives of the study were to determine the seroprevalence of RVFV in both humans and animals in Kabale and surrounding districts, identify risk factors and high-risk areas for RVFV, determine if RVF is emerging or endemic, and identify unrecognized RVF cases that may be related to the 2016 outbreak. Investigations of cattle, sheep, and goat samples from homes and villages of confirmed and probable RVF cases and from the Kabale central abattoir found that 8 of 83 (10%) animals were positive for RVFV by IgG serology; one goat from the home of a confirmed case tested also positive by RT-PCR. Whole genome sequencing from 3 clinical specimens was performed, and phylogenetic analysis inferred the relatedness of 2016 RVFV with the 2006–2007 Kenya-2 clade, suggesting previous introduction of RVFV into southwestern Uganda. An entomological survey identified 3 of 298 pools (1%) of *Aedes* and *Coquillettidia* species mosquitoes to be RVFV-positive by RT-PCR.

As stated above, VHF outbreaks tend to generate widespread media attention, including nationwide health messaging and awareness, and so most districts in Uganda have become sensitized to cases of severe disease that could be suspected VHFs. Because no RVFV had been detected in Uganda for over 40 years, the sensitivity and knowledge of this particular VHF was not as high as that of other VHFs that have received recent media attention, like EVD and MVD. Although RVF knowledge, attitudes, and practices (KAP) studies have been performed previously in Eastern Africa⁸⁹⁻⁹³, no published RVF KAP studies had been performed in Uganda. To assess what knowledge did exist for RVFV, a KAP survey was developed and administered to people living in Kabale District, near where the confirmed acute cases occurred. The Ugandan MOH, MAAIF, UVRI, and CDC initiated this study. The main objective of the study was to identify knowledge gaps in order to create programs and materials to address those needs.

3 Methods and Results:

3.1 Objective 1: Use existing molecular and serological techniques to identify and genetically characterize the first RVF outbreak identified outside of the African continent (Manuscript 1).

The first manuscript describes the identification and genetic characterization of RVFV that caused an outbreak on the Arabian Peninsula in Saudi Arabia and Yemen in 2000, this first recognized RVF outbreak outside of Africa. This was a significant event demonstrating the potential for RVF disease to spread to other regions of the world. Diagnostic testing was performed on multiple human samples collected from this outbreak to characterize the RVF cases. Laboratory detection of RVFV in samples was done with conventional molecular RT-PCR and the serological

techniques available at the time. In addition, phylogenetic analysis of selected regions of RVFV S, M, and L RNA genome segments was performed, and indicated little genetic variation among the viruses associated with disease. The Saudi Arabia and Yemen RVFV isolates were almost identical to those associated with earlier RVF epidemics in East Africa, particularly with the viruses responsible for the large RVF outbreak seen in the region in 1997–98. These results are consistent with the recent introduction of RVFV into the Arabian from East Africa.

3.1.1 Initial RVF diagnosis

In early September 2000, the MOH of Saudi Arabia received reports of unexplained hemorrhagic fever cases in the southern Tehama (coastal plain) region of south-western Saudi Arabia. Subsequently, reports were also received by the Yemen MOH of a similar disease in the adjoining Tehama region of Western Yemen. On September 15, 2000, acute-phase sera from 4 seriously ill, hospitalized patients with unexplained hemorrhagic fever were received by the VSPB, CDC, for diagnostic assessment. The shipment also contained sera from 9 close contacts of these patients, mainly household members. Based on the available clinical information, the differential diagnosis included RVFV, CCHFV, and tick-borne encephalitis- (TBE) like viruses, including Alkhurma virus. The 4 serum samples from the suspected case-patients were tested by antigen-capture enzyme-linked immunosorbent assay (ELISA) with RVFV- or CCHFV-reactive antibodies; IgM-capture ELISA with RVFV, C-CHFV, or Alkhurma virus-infected cell lysate antigens; IgG ELISA with RVFV, CCHFV, or Alkhurma virus-infected cell slurry antigen; virus isolation in Vero-E6 cells; and RT-PCR assays for detection of RVFV, CCHFV, or TBE-complex virus RNA. Evidence of RVFV infection was detected in all 4 patients with suspected cases, but no evidence of CCHFV or Alkhurma virus infection was seen. Of the 4 acute-phase serum samples, 3 were positive by RVFV antigen-capture ELISA, and the single negative sample was positive for RVFV IgM and IgG, suggesting a later stage of infection in this case. All 4 samples were positive by RVFV RT-PCR and subsequently yielded infectious RVFV upon culturing in Vero-E6 cells.

3.1.2 Viral antigen, IgM, and IgG detection in patient sera

Patient sera were tested for the presence of RVFV or CCHFV antigen, or IgM or IgG antibodies reactive with these viruses and Alkhurma virus, a member of the TBE complex that was discovered in Saudi Arabia⁹⁴. RVFV and CCHFV antigen-capture assays were performed in an ELISA format essentially as described⁹⁵. The RVFV assay used polyclonal hyperimmune ascitic fluid raised against RVFV strain Zagazig 501 as the capture antibody, and rabbit hyperimmune serum raised against RVFV Zagazig 501 as the detector antibody. The CCHFV assay used a sheep hyperimmune serum raised against a South African CCHFV strain as the detector antibody, and a mouse hyperimmune ascitic fluid raised against CCHFV strain IbAr10200 as the capture antibody. IgM antibody titers were determined by IgM antibody-capture ELISA, with RVFV, CCHFV, or Alkhurma virus-infected cell slurry prepared as described⁹⁵. IgG antibody titers were determined using RVFV, CCHFV, and Alkhurma virus-infected cell antigens in an ELISA format similar to that described previously⁹⁵.

3.1.3 Virus isolation and RNA extraction

Viral RNA was obtained directly from patient blood or serum samples collected during the outbreak or from virus isolated from patient serum that was passaged once in Vero-E6 cells. A viral stock was prepared by placing 100 µL of serum (sample 200010901) onto a confluent monolayer of Vero-E6 cells in a T-25 flask, and 200 µL of passage 1 cell supernatant was placed into 1 mL of TriPure for RNA purification. Saudi Arabia sample 2003043 and Yemen sample 2001373 were prepared by placing 200 µL of blood or serum, respectively, directly into 1 mL of TriPure. RNA was extracted onto glass beads by using RNAid kit according to a modified protocol.

Of the 9 serum samples from close contacts, 2 showed evidence of RVFV infection. One was positive by RT-PCR and RVFV isolation, and the other contained RVFV IgM antibodies.

A second shipment, which arrived at CDC on September 20, 2000, contained acute-phase sera from an additional 15 hospitalized patients and 12 contacts; 13 of 15 suspected patients had evidence of RVFV infection. Four contacts of case-patients also showed evidence of RVFV infection, thus confirming that RVFV was responsible for the outbreak on the Arabian Peninsula.

3.1.4 Detailed genetic analysis

RNA extracted from 3 representative viruses was chosen for more detailed genetic analysis. Included was RNA from RVFV isolate (strain 200010901) obtained from the first RVFV-infected patient to be laboratory-confirmed during the current outbreak and representing the early phase of the outbreak in Saudi Arabia. This isolate was obtained from a serum sample collected on September 13, 2000, from this patient, who was infected in Jizan Province. RNA extracted from a serum sample collected late in the outbreak in Saudi Arabia was also included; this serum sample (2001373) was collected on November 22, 2000, from a patient infected in Asir Province. The third RNA sample was extracted from a blood sample obtained from a patient in Yemen (sample 2003043). These RNA samples were compared to detect any genetic variations in the RVFV strains active during the early and late phases of the outbreak in Saudi Arabia and to evaluate whether the same virus strain was responsible for disease in both Saudi Arabia and Yemen.

A single nucleotide difference was observed between each of the S RNA segment fragments (601 nt) analyzed from these Saudi Arabia and Yemen samples. Similarly, no nucleotide differences were found between Saudi Arabia 200010901 and Yemen 2003043 virus M RNA segment fragments (510 nt) analyzed, and only 1 nt difference between these samples and the Saudi Arabia 2001373 M segment. The L RNA genome segment fragment analyzed (129 nt) was identical in all 3 viruses. These data demonstrate that the viruses in the early and late stages of the RVF outbreak in Saudi Arabia were identical to one another and to the virus causing disease in Yemen.

The results of phylogenetic analysis of the nucleotide sequence differences between the S, M, and L RNA genome fragments of the Saudi Arabia and Yemen viruses and previously described RVFVs are shown in Figure 10. Earlier maximum likelihood analyses had separated RVFVs into 3 broad groups, which predominantly contained viruses from North Africa, West Africa, and East/Central Africa⁹⁶. All 3 RNA segment trees obtained grouped the Saudi Arabia and

Yemen viruses with the East/Central Africa viruses. Specifically, the S, M, and L RNA genome segments of the Saudi Arabia and Yemen viruses were closely related to those of viruses previously detected in outbreaks in East Africa, as represented by the Kenya 1997 and Madagascar 1991 virus isolates (Figure 10). The nucleotide changes in the S, M, and L RNA genome segment fragments observed among the closely related Saudi Arabia/Yemen viruses and the Kenya 1997 and Madagascar 1991 viruses were synonymous changes, resulting in no amino acid differences among these isolates.

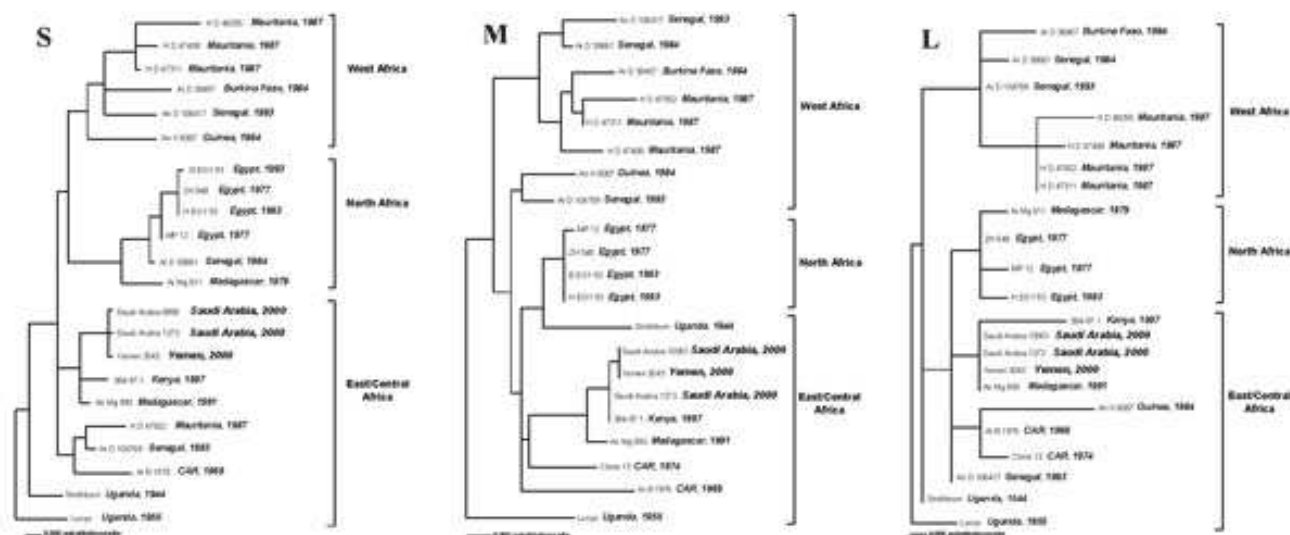


Figure 10: Phylogenetic relationship of the S, M, and L RNA segments of RVFV. Maximum likelihood analysis of the nt sequence differences in a 661 nt region of S RNA segment (Panel A), a 708 nt region of the M RNA segment (Panel B), and a 129 nt region of the L RNA segment (Panel C) of RVFVs was performed using PAUP4.0b10.⁸

The rapid RVFV RT-PCR assay was a useful complement to the RVFV antigen and IgM-capture ELISA tests for diagnosis of acute illness, as it detected viral RNA in 15 of 16 serum samples that were subsequently found to be RVFV-positive by other methods. Overall correlation between the various RVFV diagnostic assays was good. Nucleotide sequence analysis of the 186 nt (excluding primer regions) PCR products amplified from these initial specimens confirmed the virus identity as RVFV and showed no nucleotide differences between the viruses detected in these Saudi Arabian patients.

3.2 Objective 2: Develop and validate a new sensitive and broadly reactive quantitative RT-PCR assay for rapid laboratory detection of RVFV (Manuscript 2).

The next manuscript aimed to improve existing RVFV diagnostic assays and to develop a broad-based real-time RT-PCR molecular diagnostic assay. This manuscript details the development of a real-time quantitative RT-PCR (qRT-PCR) assay with high nucleotide conservation of both primer and probe regions for a total of 40 RVFV strains that,

when coupled with high-throughput 96-well RNA extraction methods, allows for the rapid identification of infected patients and animals. This assay has demonstrated its capability for highly sensitive and efficient detection of RVFV from human clinical materials utilizing archived patient samples collected in Saudi Arabia during the previously described RVFV outbreak of 2000. The rapid and high-throughput detection of RVFV in human and livestock samples was a critical first step in the identification and eventual control of this significant pathogen in future outbreaks.

3.2.1 Primer and probe design and standard curve optimization

Full-length complete genome sequences of the S, M, and L RNA segments of 33 biologically and phylogenetically diverse RVFV isolates identified previously⁷⁷, plus 7 additional RVFV strains, were aligned and analyzed. A highly conserved genomic domain was identified within the L segment, spanning nucleotides 2800 to 3200. Located within this region were several potential forward and reverse primer and probe annealing sites that obeyed basic real-time RT-PCR optimization guidelines with minimal nucleotide mismatches. To determine which of these potential primer-probe combinations were optimal for detecting RVFV, in vitro RNA transcripts of the L gene segment encompassing the annealing sites of these primers and probes were amplified from a cDNA plasmid containing the entire L segment of RVFV strain ZH-501 (described previously⁷⁷). This in vitro-transcribed and purified RNA was then serially diluted 10-fold (10:1 to 10:15) in nuclease-free H₂O to generate a standard curve to compare the relative sensitivities of various potential primer-probe combinations and reaction conditions. Each serial dilution was run in replicates of 8 for qRT-PCR amplification.

3.2.2 Analytical sensitivity and specificity determination using stock RVFV

After optimizing the primer and probe composition and reaction conditions with in vitro-transcribed RNA, the analytical sensitivity and efficiency of this assay were verified by using RNA extracted from wild-type RVFV strain ZH501 and serially diluted in a variety of diluents. The diluents included normal human control serum and cell culture medium commonly used in the preparation of RVFV stocks. Stock RVFV strain ZH501, 1.7×10^6 PFU/mL, was serially diluted 10-fold to establish a standard curve in each respective diluent and then extracted in replicates of 8 on a 6100 nucleic acid purification platform (Applied Biosystems) as described elsewhere⁹⁷. The limit of detection (LOD) was determined as the final dilution for which a cycling threshold value (C_T) was determined. To ensure that phylogenetically distant RVFV strains could be detected by this assay, RNA extracted from 21 diverse RVFV strains collected over a period of 56 years (1944 to 2000) and representing all known RVFV lineages was amplified by qRT-PCR according to the above protocol. In addition, to determine whether non-specific annealing might yield false-positive results in total cellular RNA extracted from potential host species, the total RNA of tissue samples from bovine, ovine, human, rodent, and mosquito species was tested according to the above qRT-PCR protocol.

3.2.3 Q-RT-PCR assay validation with human clinical materials

The human clinical specimens used in this study were collected during an outbreak of RVF in Saudi Arabia in 2000⁸. A total of 62 patient acute-phase blood samples, collected from a total of 33 patients (25 with non-fatal and 8 with fatal outcomes), were tested. Of these, a total of 26 samples were single blood collections from 26 infected patients (20 non-fatal and 6 fatal outcomes) with a known date of collection after onset of clinical symptoms. A subset of samples (36) contained serial blood collections from a total of 7 patients (5 non-fatal and 2 fatal outcomes) that were obtained on multiple days after the onset of clinical symptoms until either the patient's recovery or death. For all specimens tested, the final outcome for each patient and day of sample collection after symptom onset were known. For validation purposes, the results from this pan-RVFL qRT-PCR were compared to earlier results of antigen capture and IgM and IgG serological testing^{8, 69}. A significant difference was found in patient RVFL RNA load (as reflected by mean qRT-PCR C_T values) between fatal and non-fatal cases at days 1 to 4 (mean C_T values: fatal = 19.4, non-fatal = 31.2; $p < 0.001$), days 5 to 8 (mean C_T values: fatal = 24.5, non-fatal = 35.8; $p < 0.001$), and days 9 to 14 (mean C_T value: non-fatal = 35.9; $p < 0.001$) after the onset of clinical symptoms (Figure 11). Among non-fatal cases, significant decreases in patient RVFL RNA load were detected between days 1 to 4 and between days 5 to 8 ($p < 0.05$) post symptom onset. Interestingly, in non-fatal cases, samples collected 5 to 14 days after onset of symptoms were often positive only for the presence of RVFL-specific antibodies or demonstrated very low levels of RVFL RNA, presumably reflecting the clearance of virus from patient blood to levels below the qRT-PCR detection threshold (Figure 11).

After analyzing the complete S, M, and L segments of 40 biologically and ecologically diverse RVFL strains, a highly conserved region was identified on the virus L segment at approximate nt positions 2800 to 3200. Careful analysis of this region identified multiple potential annealing sites of primer and probe combinations that followed basic rules of real-time PCR design. A total of 6 sets of primers/probes spanning this region were designed and tested. The set RVFL-2912fwdGG, RVFL-2981revAC, and RVFL-probe-2950 demonstrated the greatest analytical sensitivity and efficiency and highest nucleotide conservation. Using serial 10-fold dilutions of transcribed RNA to establish a standard curve, the LOD of the optimized qRT-PCR assay was determined to be approximately 5 genome copies per reaction, and this set yielded a slope of -3.58 when C_T values were plotted versus \log_{10} dilutions of RNA. A total of 21 RVFL isolates was tested to ensure detection of diverse RVFL strains. Using total RNA extracted from infected cell culture supernatants, all 21 phylogenetically diverse strains were detected by the above primer/probe set. No false-positive or off-target results were generated due to non-specific annealing with the total RNA of several potential host species (bovine, ovine, mouse, rat, mosquito, and human).

Using purified in vitro-transcribed RNA from the RVFL L segment as a template, reaction conditions were optimized to determine the LOD of this assay. Multiple experiments comparing single-step (RT and quantitative PCR in a single tube) or 2-step (RT and quantitative PCR in separate tubes) assays showed that the overall analytical sensitivity was reduced using a single-step protocol.

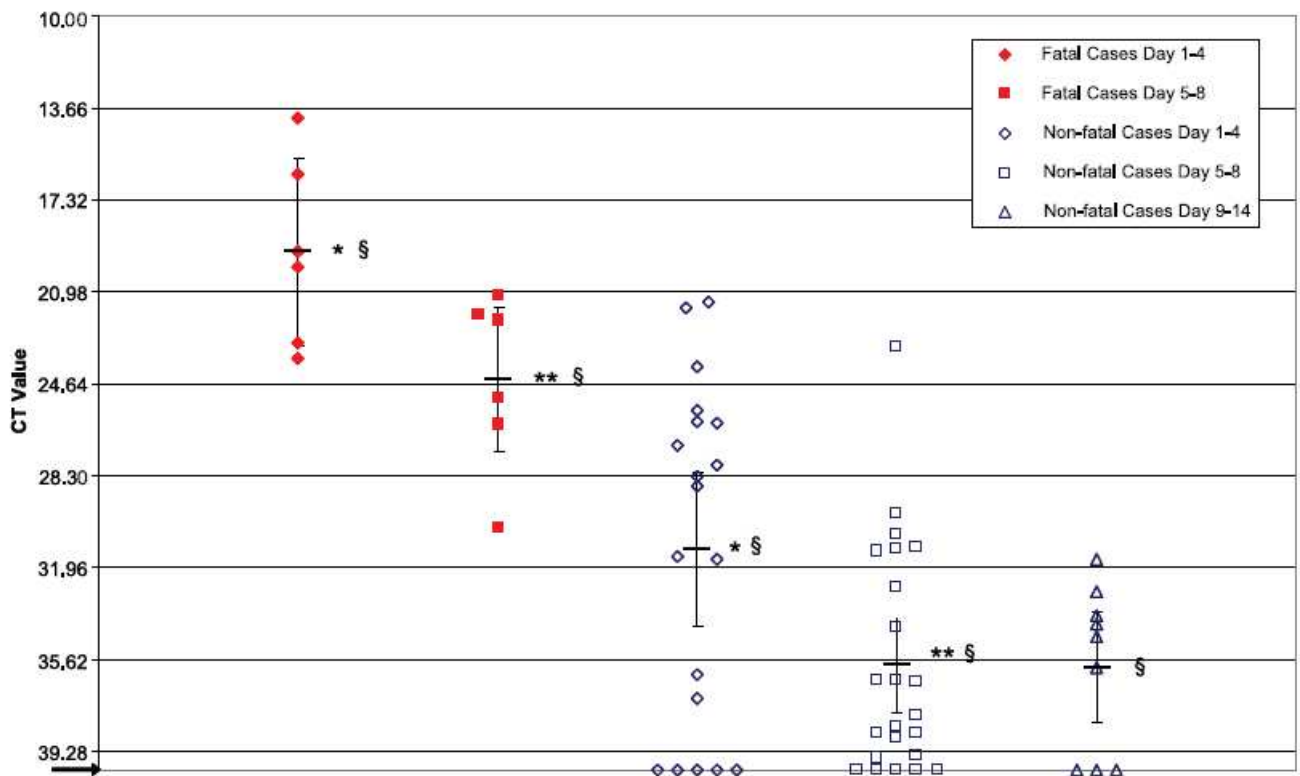


Figure 11: RVFV RNA loads in patients with fatal and non-fatal outcomes. Results of qRT-PCR amplification of RVFV-infected patient serum samples collected during the epidemic in Saudi Arabia in 2000. A total of 62 patient specimens were divided into groups according to outcome (non-fatal or fatal) and by days after onset of clinical symptoms (days 1 to 4, 5 to 8, and 9 to 14). Significant differences ($p < 0.001$) between the mean C_T values of fatal and non-fatal groups 1 to 4 days post infection are indicated by a single asterisk (*), and at 5 to 8 days by a double asterisk (**). At all timepoints, the mean RVFV RNA load of fatal cases was significantly higher ($p < 0.001$) than in non-fatal cases and is indicated by \$. Note that the mean serum RVFV RNA loads of both fatal and non-fatal cases decrease as the time after symptom onset increases, presumably due to the development of anti-RVFV IgM and/or IgG antibodies and subsequent clearance of virus from blood. Error bars indicate the mean value ± 2 standard errors of the mean. The LOD of the qRT-PCR assay is indicated by an arrow and represents a C_T value of >40 . The scale of the y-axis scale is demarcated every 3.66 C_T values, which corresponds to a $1.0 \log_{10}$ PFU/ μ L change in viral RNA titers as calculated by titrating stock RVFV.⁹

To investigate the dynamics of qRT-PCR and serologic assay results relative to time after symptom onset, serial samples collected from 5 patients with non-fatal and 2 patients with fatal outcome were analyzed⁸. The results from 4 representative patients (2 non-fatal and 2 fatal) are illustrated in Figure 12. As expected, and in concordance with the serologic assays, the qRT-PCR assay correctly identified the specimens from acutely infected patients. Among the serially sampled non-fatal cases, IgM and IgG adjusted optical density (SUM_{OD}) values increased over time, while a corresponding decrease was observed in both RVFV antigen capture SUM_{OD} and RNA load, as measured by qRT-PCR C_T values. In these non-fatal cases, RVFV antigen capture values decreased to negative levels at least 5 days prior to the loss of detectable qRT-PCR signals. In all non-fatal cases, both RVFV antigen capture and qRT-PCR values began to

decrease (days 1 to 4) before significant increases in RVFV-specific IgM and IgG were detected, a result likely due to the lack of circulating free anti-RVFV IgM or IgG available for detection early in the course of infection. In contrast, the 2 serially sampled fatal cases had extremely high RVFV antigen capture and low qRT-PCR C_T values 3–5 days after onset of illness, with no significant levels of anti-RVFV-specific IgM or IgG. Importantly, the viral loads in these fatal cases (as measured by RVF antigen capture and qRT-PCR) did not significantly decrease during the follow-up period.

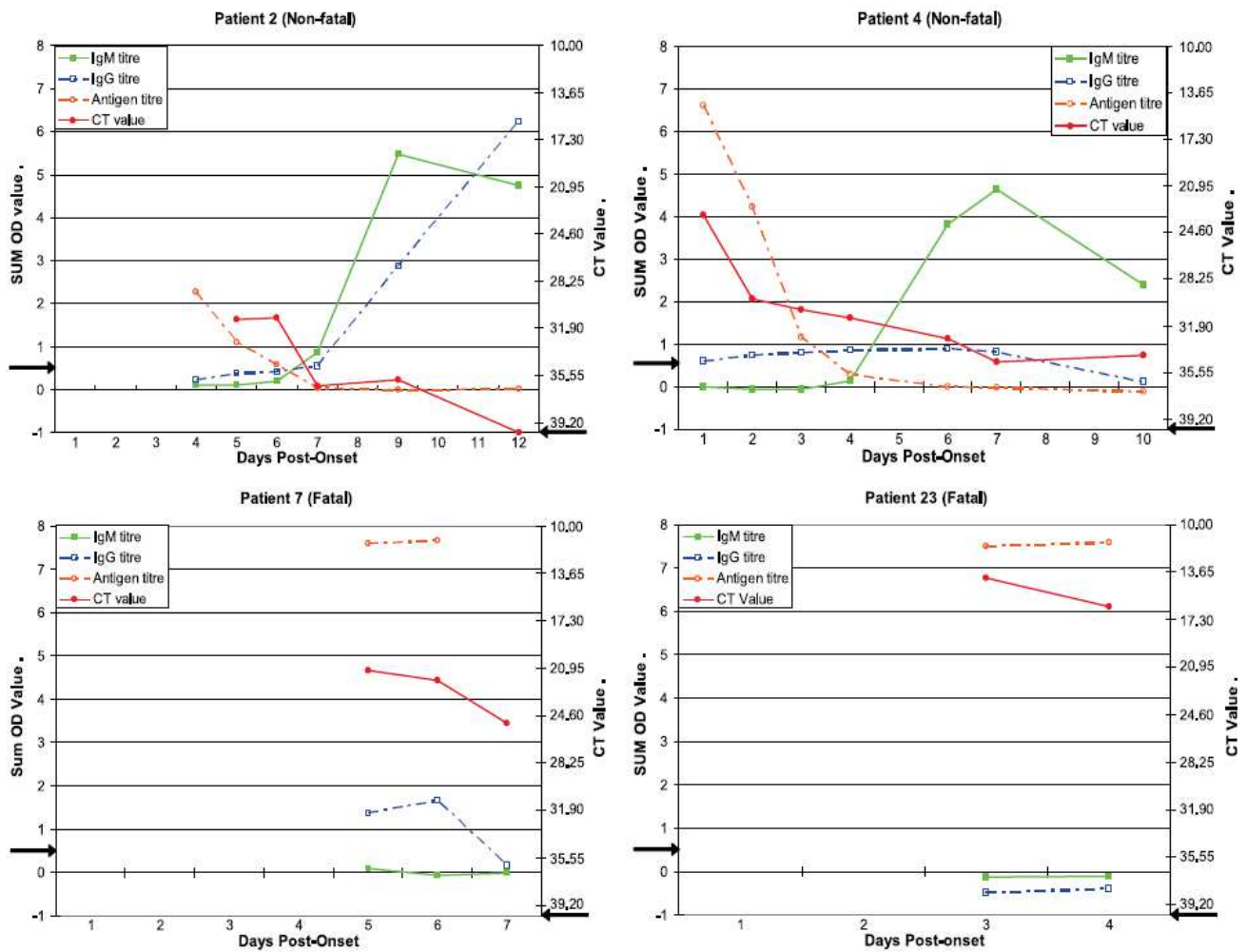


Figure 12: Results of qRT-PCR and RVFV-specific antigen capture and IgM, and IgG ELISAs of a representative subset of 4 human patients from whom multiple serial blood samples were obtained. Patients 2 and 4 had non-fatal outcomes, while patients 7 and 23 died. IgM, solid square and solid line; IgG, open square and dashed line; antigen capture, open circle and dashed line; qRT-PCR C_T values, closed circle and solid line. All serological data are expressed as the adjusted SUM_{OD} of 4 sample dilutions of 1:4, 1:16, 1:64, and 1:256 for the anti-RVFV antigen capture assay, and dilutions of 1:100, 1:400, 1:1,600, and 1:6,400 for the anti-RVFV IgM and IgG assays. The LOD of the qRT-PCR assay is indicated by an arrow and represents a C_T value of >40 . The scale of the right-hand y-axis scale is demarcated every 3.66 U, which corresponds to a $1.0 \log_{10}$ PFU/ μ L change in viral RNA titers as calculated by titrating stock RVFV.⁹

3.3 Objective 3: Design and implement a comprehensive surveillance system in Uganda to rapidly detect, respond to, and control VHF outbreaks (Manuscript 3).

Manuscript 3 details events that follow the establishment of the national VHF surveillance system in Uganda and describes the first VHF outbreak detection of single human case of SUDVs. Within 2 years of detecting this first outbreak, 4 more filovirus outbreaks were detected, confirmed, and investigated by the Uganda national VHF surveillance system, including 2 outbreaks of SUDV and an MVD outbreak in Uganda, and confirmation of BDBV in eastern DRC. The high frequency in filovirus outbreaks detected in such a short time caused international concern for potential genetic changes in these viruses that could lead to more frequent outbreaks.

The Uganda national VHF surveillance and laboratory program was established in 2010 as a collaboration between UVRI, Uganda MOH, and the CDC's VSPB. It was a first of its kind national VHF sentinel surveillance system, established for the purposes of enhancing Uganda's Integrated Disease Surveillance and Response system to rapidly detect, diagnose, report, and respond to VHFs and other emerging zoonotic infectious diseases. In addition, the program was to expand and enhance laboratory diagnostic testing for VHFs in Uganda, improve incident case detection through better clinical recognition, and use the generated data to better inform decision-making and disease control efforts at the national level.

This comprehensive program was implemented through the creation of a series of sentinel surveillance sites directly linked to a newly created high-containment laboratory at the Uganda UVRI in Entebbe. The program initially had 6 directly supported sentinel surveillance sites and has now grown to over 20 sites (Figure 13). Regional hospital and district health office staff were trained in epidemiological data collection, VHF surveillance, epidemiology and ecology, suspect patient identification, and infection control procedures. The primary VHF surveillance protocol consists of a standardized 3-tiered (suspect, probable, and confirmed) case definition for VHFs, a standardized case reporting form, and provision of supplies to collect and submit clinical samples for virologic testing at the Uganda National Viral Hemorrhagic Fever Laboratory at UVRI in Entebbe. Results are reported to Uganda MOH. Each site is supplied with a cabinet containing the following materials: vacutainer tubes (EDTA), butterfly needles, disposable personal protective gowns, eye protection, N95 masks, latex gloves, zippered sample bags, insulated sample transport containers, ice packs, and VHF case report log books. All data collected by the VHF surveillance program are entered into an electronic database for review and analysis at the national level at UVRI. In addition, both UVRI and CDC technical staff regularly visit the sites to conduct refresher training, restock supplies and materials, and provide technical assistance to surveillance staff.

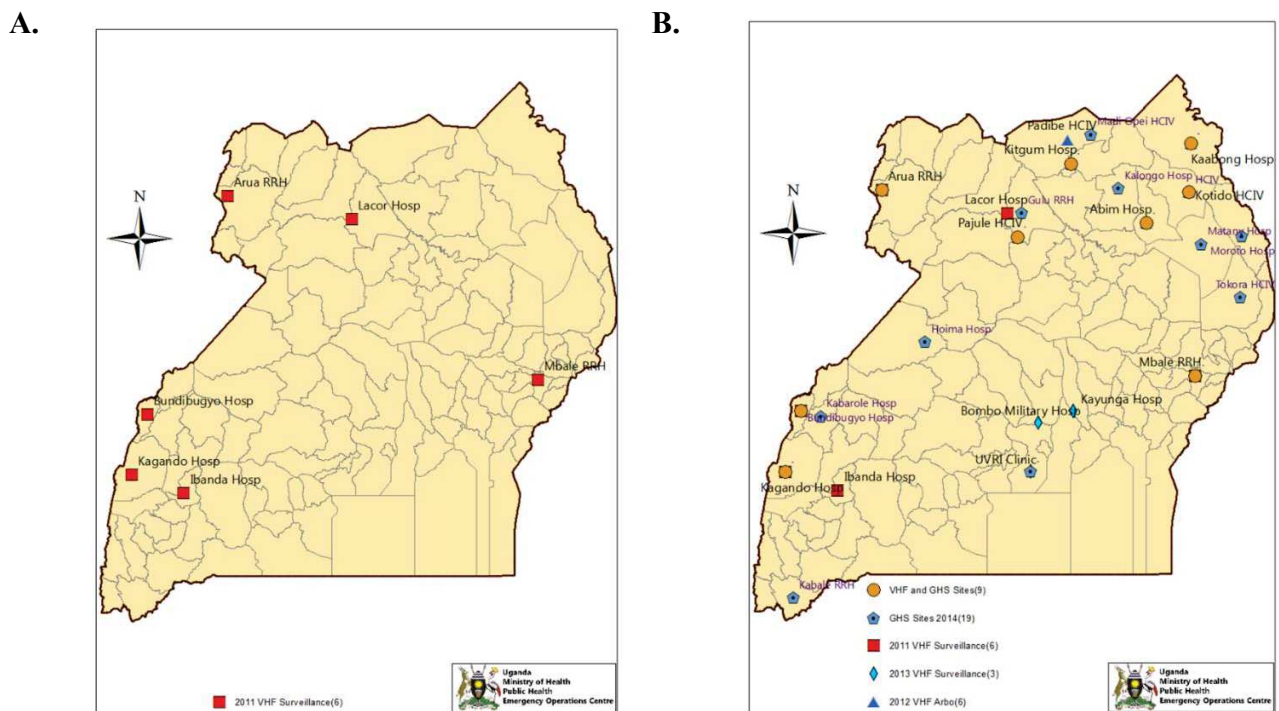


Figure 13: Uganda VHF sentinel surveillance sites in 2011 (A) and in 2017 (B). (unpublished figure)

The UVRI VHF program also works with many national and international partners to initiate new surveillance activities and perform prospective and retrospective studies and serosurveys. One example is a collaboration with the Karamoja veterinary laboratory in Moroto, eastern Uganda. The UVRI VHF program collected blood samples from bovine, caprine, ovine, and porcine species, and tested over 1500 samples for RVFV, CCHFV, and other arboviruses by serology. Additionally, as a follow-up surveillance activity to the 2012 MVD outbreak in Ibanda and Kibaale, the VHF program conducted a serosurvey in high-risk populations in Ibanda and Kamwenge districts. The program also analyzed possible past VHF infection by using 4 previously completed national surveillance surveys and serosurveys: the Uganda AIDS Indicator Survey, the Yellow Fever Risk Assessment survey, WHO Expanded Program on Immunization rash surveillance, and UVRI hepatitis surveillance.

The VSPB of the CDC has provided the UVRI VHF laboratory with all needed laboratory diagnostic test reagents to enable confirmation of suspected VHF cases from clinical samples sent in from district health centers and hospitals. These include test reagents and positive and negative controls for confirming a number of infections, including EVD, MVD, RVFV, and CCHFV. The diagnostic tests available are antigen-detection ELISA, IgM and IgG ELISA, and real-time RT-PCR. All VHF confirmatory testing is performed at UVRI and results are available within 24–48 h after sample reception.

All testing is performed in the newly renovated biosafety level 3 high-containment suite. Standard operating procedures and laboratory quality management protocols have been developed and adapted to ensure that all samples are safely processed, inactivated, and tested with minimal manipulation and risk of exposure to laboratory staff. Enhanced biosafety practices are strictly followed and proper engineering controls are in place to ensure maximum protection. Enhanced biosecurity controls have also been established to ensure limited access to restricted areas and the high-containment suite.

After this national VHF surveillance system was established, on May 6, 2011, a 12-year-old girl from Nakisamata Village, Luweero District, was admitted to Bombo Military Hospital. The patient presented with fever, jaundice, and hemorrhagic signs, including epistaxis, hematemesis, hematuria, and conjunctival, gingival, and vaginal bleeding. The attending physician made a preliminary diagnosis of disseminated intravascular coagulopathy with a functional platelet disorder, along with VHF as a potential etiology. The patient was therefore promptly isolated from the general ward and other outpatients. Hospital staff involved in her care and treatment implemented isolation precautions, including the use of protective gowns, gloves, and masks. The patient's condition worsened and, despite tracheal intubation and supplemental oxygen, she died 3 h after admission. Because the cause of her death was still unknown but VHF was suspected, her body was disinfected using chlorine solution, wrapped in plastic, and then taken to the hospital mortuary facility where she was placed in a sealed coffin. The coffin was handed over to her relatives for burial the following day; they were instructed not to open it or touch the body in any way prior to burial.

A blood sample collected from the patient at the hospital prior to her death was transported to the CDC/UVRI laboratory for diagnostic testing by RT-PCR, antigen detection ELISA, and IgM for filoviruses^{79, 95, 97-99}. SUDV was detected by RT-PCR and confirmed by antigen detection ELISA. IgM ELISA for EBOV and all tests for MARV were negative. SUDV was also isolated from Vero-E6 cells at the VSPB in Atlanta, USA. Overlapping PCR fragment copies of the complete viral genome were amplified and the nucleotide sequence was obtained as described previously¹⁰⁰. Maximum-likelihood phylogenetic analysis confirmed SUDV and demonstrated the isolate (Nakisamata isolate, JN638998) to be closely related (99.3% identical) to the Gulu SUDV strain obtained from northern Uganda in 2000 (Figure 14).

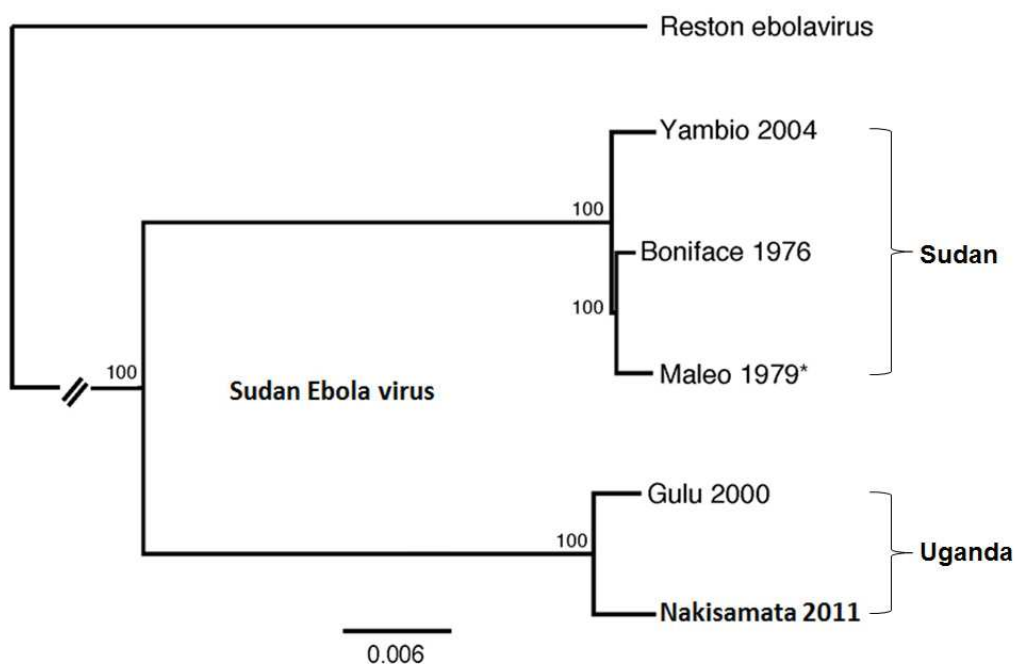


Figure 14: Maximum likelihood tree obtained from full-length sequences of SUDV strains Nakisamata (JN638998), Boniface (FJ968794), Gulu (AY729654), and Yambio (EU338380), and the polymerase gene (*) of Maleo (U23458); full-length RESV (AY769362) is included as an outgroup. Bootstrap values listed at the nodes provide statistical support for 1,000 replicates. Scale bar indicates 0.006 substitutions per site.¹⁰

On May 13, an investigation team traveled to Bombo Military Hospital and Nakisamata Village, the home of the index case; the village is located in Luweero District, approximately 50 kilometers north of Kampala. The investigation team established that the index case (above-described 12-year-old female) started feeling ill on May 1, 2011, complaining of a mild headache for which she was given an over-the-counter analgesic. She developed a fever accompanied by chills on May 4, and began vomiting the next day. On May 6, she had developed intense fatigue and epistaxis. Her grandmother then took the girl to a local health clinic, where she received adrenaline nasal packs for her epistaxis and 2 injections of quinine and vitamin K. However, her condition continued to worsen and she developed hematemesis as well as vaginal bleeding. She was then transported by motorcycle taxi to Bombo Military Hospital, approximately 35 kilometers north of Kampala, by her grandmother and father.

Relatives reported that the index patient did not travel outside Nakisamata in the 3 months preceding her illness, recalled no unusual deaths in the area in recent months, and said she did not attend any funerals or have contact with anyone visiting from another town or village prior to her illness. They also reported that she had no exposure to any sick or dead animals in the village or nearby forested area. Samples from 4 family members, none of whom reported illness, were obtained and tested for SUDV by RT-PCR, antigen detection ELISA, and IgM

and IgG ELISA. All were negative with the exception of samples from one juvenile relative, who had IgG titers of 1:1600, indicating past infection with SUDV. Since IgM antibodies persist for up to 2 months after infection^{95, 101}, this person's infection appears temporally unrelated to that of the 12-year-old index patient. No clinical information was available for this person to determine if this infection was symptomatic, as contact studies and serosurveys suggest some ebolavirus infections may go unrecognized^{95, 102-104 105}.

The investigation team identified 25 close contacts, including 13 individuals who had physical contact with the index patient after illness onset at her home and 12 hospital staff. Four of the hospital contacts were classified as having high-risk exposure because of potential exposure to bodily fluids, including 2 hospital staff who performed tracheal intubation and 2 who handled the body following death. During outbreak response and follow-up surveillance (21 days after death of the index patient), 24 more sick individuals, including 18 from Luweero District and 6 from other locations in Uganda, were identified, but EVD was subsequently ruled out by laboratory testing at UVRI.

The successful implementation of the VHF surveillance program allowed detection and laboratory confirmation of multiple VHF outbreaks. Since this initial EVD outbreak detection and response in 2011¹⁰, the UVRI VHF surveillance and laboratory program has tested over 15,000 clinical samples from surveillance activities, serosurveys, and VHF outbreaks. The samples tested confirmed over 30 independent VHF outbreaks, including 4 EVD outbreaks in 2011¹⁰ and 2012; 3 MVD outbreaks in 2012³², 2014³³, and 2017¹⁰⁶; multiple independent outbreaks of CCHFV in 2013 and 2015¹⁰⁷ to present; and outbreaks of RVFV beginning in 2016¹³ to present (Figure 15). Samples have been tested from most districts throughout Uganda, and reference testing was provided to 6 East and Central African countries. The laboratory is now the official Uganda national VHF reference laboratory, as well as an East Africa regional VHF reference laboratory. During the BDBV outbreak in Isiro, DRC, in 2012, the CDC-UVRI VHF laboratory served as the base of operations for outbreak response and diagnostic testing. On a case-by-case basis, the laboratory is able to provide on-site mobile PCR testing capability during outbreaks, greatly decreasing the time from admission of suspect patients into the isolation ward and initial confirmation of acute infection. This capability was utilized during the MVD outbreak in Kabale and Ibanda Districts in 2012, and recently in Kasese District to support testing of suspected cases imported from neighboring DRC in response to the ongoing EVD outbreak in that country.

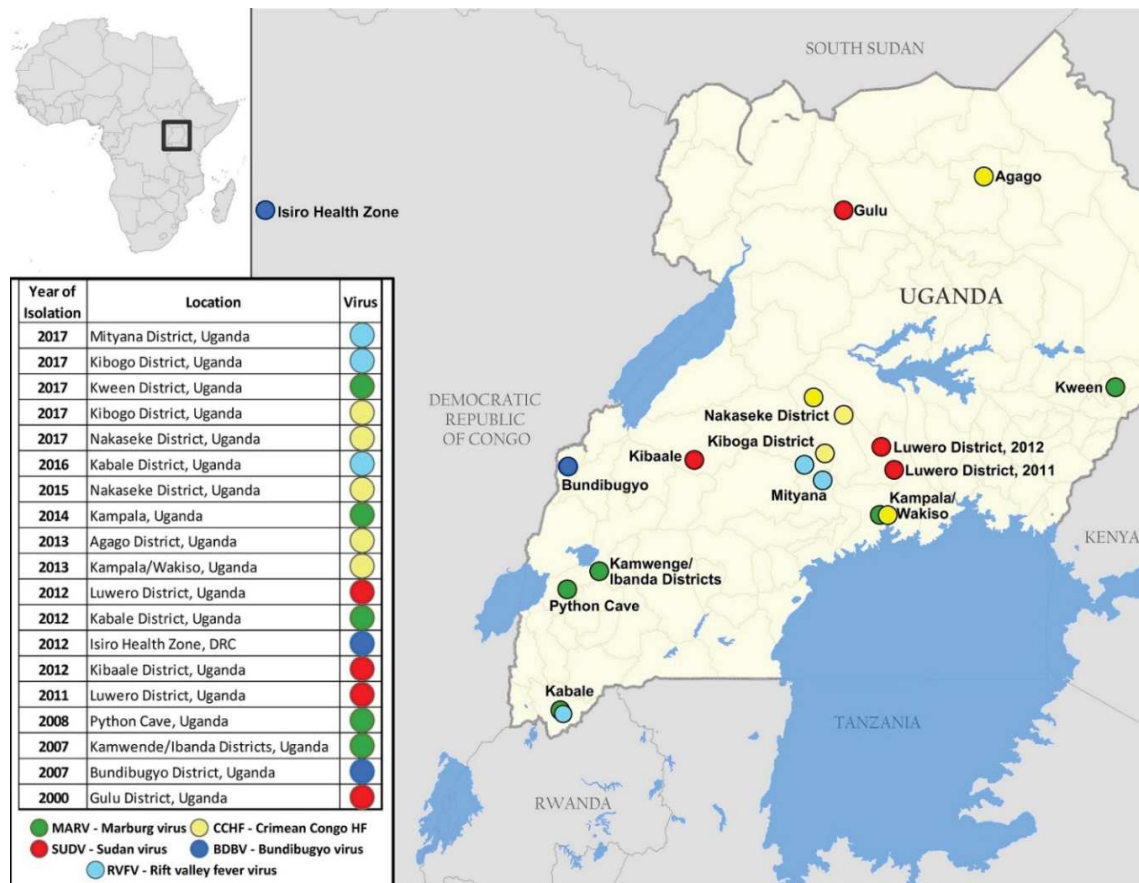


Figure 15: Map showing VHF outbreaks identified and confirmed in Uganda from 2000 to 2017¹

3.4 Objective 4: Describe the effectiveness of the Uganda VHF surveillance system and quantify key response and control indicators (Manuscript 4).

Following the successful implementation of a national VHF surveillance system in Uganda, including the detection of 11 independent VHF outbreaks between 2011 and 2017, this manuscript describes the positive impact of this surveillance system on rapid detection and response to VHF outbreaks. The number of detected outbreaks increased 5-fold from the previous 10 years once VHF surveillance was implemented. At the same time, the overall intensity and duration of each outbreak was dramatically reduced, as the time between initial clinical notification and laboratory confirmation was shortened from an average of 2 weeks prior to 2010, to an average of just 2.5 days. Earlier case detection and laboratory confirmation have led to faster public health responses, including earlier patient isolation, implementation of infection control precautions^{10, 32, 33}, decreased secondary transmission in the communities ($p = 0.01$)^{10, 20, 32, 33, 78}, and ultimately shorter ($p < 0.0001$) and less severe outbreaks ($p = 0.001$). Additionally, significant reductions in overall morbidity ($p = 0.001$) and mortality ($p = 0.01$) from continued VHF outbreaks has been observed following surveillance implementation (Figure 16). For comparison, 2 large outbreaks of SUDV and BDBV occurred in

2000⁷⁸ and 2007²⁰, respectively, during an era of limited surveillance and outbreak detection capacity. Uganda’s VHF surveillance system serves as a model for detecting and responding to public health threats of international concern.

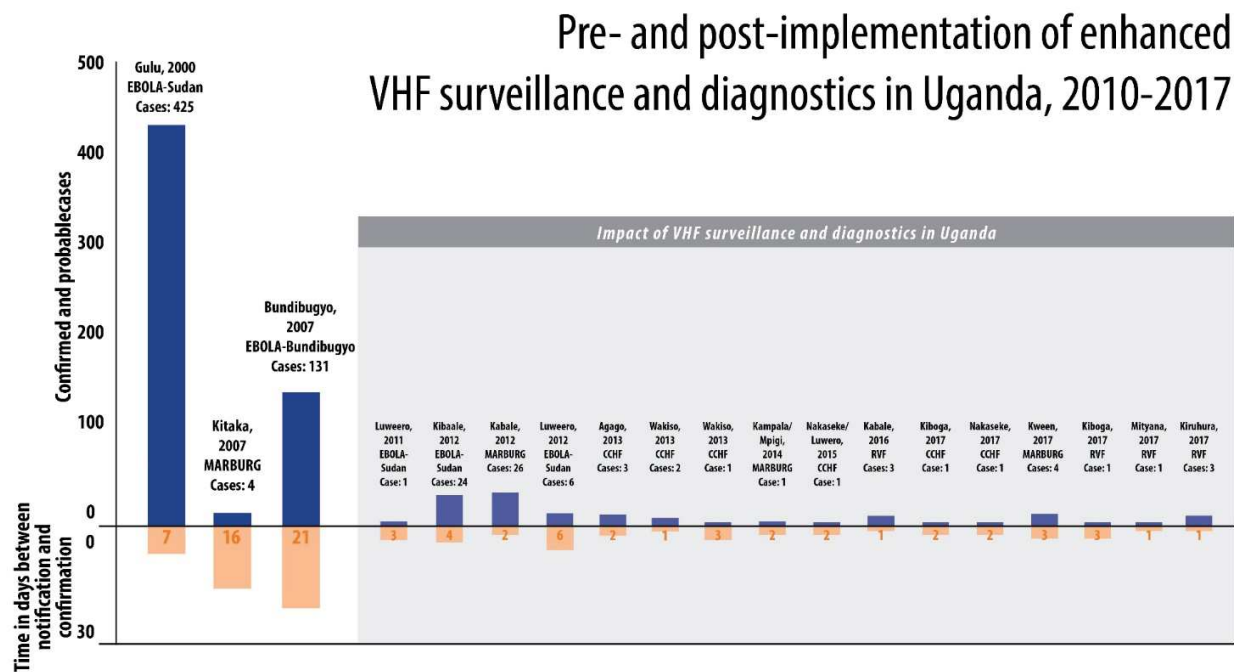


Figure 16: Impact of enhanced VHF surveillance in Uganda on outbreak detection and outbreak magnitude and scope relative to the reduction in time from notification to laboratory confirmation (2011–2017)¹¹

3.5 Objective 5: Genetically compare filovirus isolates obtained from Uganda VHF surveillance and analyze for potential genetic virulence factors contributing to their continued re-emergence (Manuscript 5).

The next manuscript compared the genetic sequences of the newly identified outbreak-involved strains of SUDV, BDDV, and MARV to previously isolated outbreak strains of SUDV, RESV, EBOV, TAFV, BDBV, MARV, and Ravn virus, concluding that no significant genetic changes occurred that would have increased virulence or pathogenicity to account for the higher outbreak frequency. The UVRI VHF surveillance program identified and laboratory-confirmed 4 independent filovirus disease outbreaks between July and October of 2012 by testing clinical samples via antigen capture, IgM ELISA, and/or qRT-PCR, using previously described methods^{10, 79, 100}. The VSPB subsequently isolated the viruses and completed viral genome sequencing studies. Samples from patients with fatal and non-fatal outcome that tested positive by qRT-PCR and showed low C_T values were chosen randomly for sequencing full viral genomes.

In July 2012, samples from VHF alert cases in Kibaale District of western Uganda were received at the UVRI VHF laboratory for testing. Differential molecular testing of clinical samples identified the outbreak as EVD associated with SUDV. Definitive identification of the virus as SUDV was done at CDC by sequencing the complete genome of the virus directly from the clinical samples. Genomic analysis showed ~99.9% sequence identity among the virus genomes detected in the 4 acute case serum samples. Finding nearly identical virus sequences is compatible with a single spillover event from the virus reservoir into the human population, with subsequent limited waves of human-to-human transmission. Interestingly, these sequences also showed a high identity (~99.2%) with the above-mentioned SUDV Nakisimata isolate¹⁰ detected in 2011 in Luweero District, and with the SUDV isolate¹⁰⁸ from the Gulu District of Northern Uganda in 2000 (Figure 17). By the end of the outbreak (officially declared on October 4th, 2012), a total of 11 cases had been confirmed by molecular and serological testing.

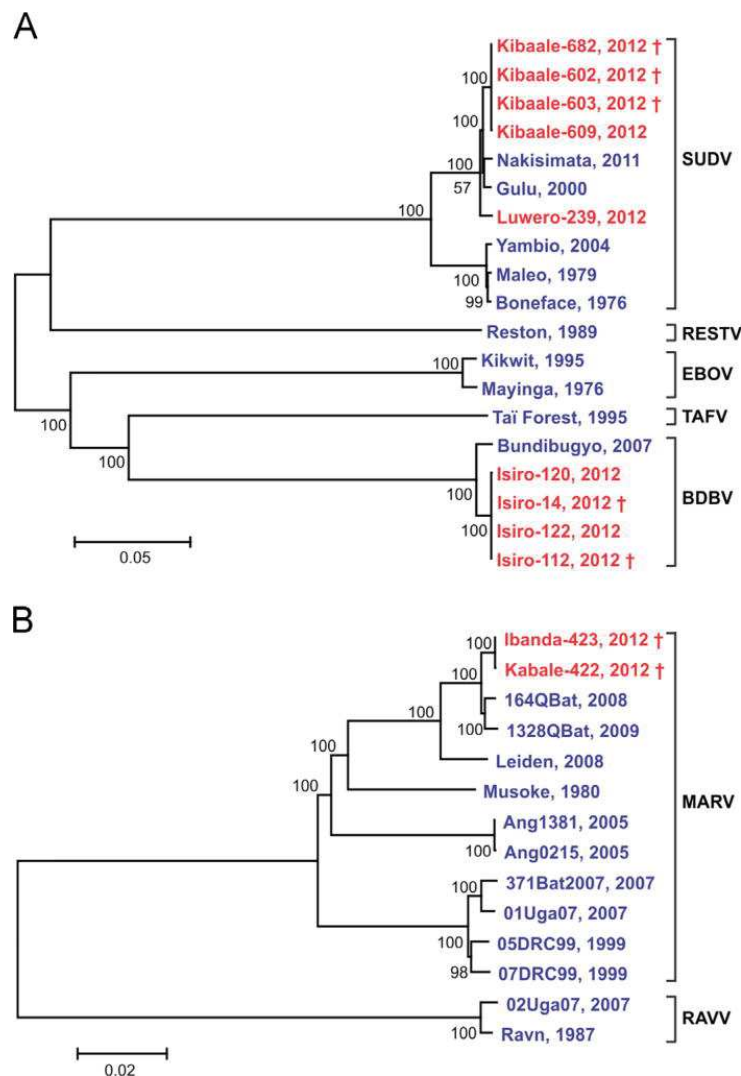


Figure 17: Phylogenetic trees comparing representative full-length genomes of (A) ebolaviruses and (B) MARV. Evolutionary analyses were conducted in MEGA5 using the neighbor-joining method. Bootstrap values listed at the nodes provide statistical support for 500 replicates. Scale bar indicates the number of substitutions per site.¹²

In mid-August 2012, samples from VHF alert cases in Isiro and Dungu health zones in northeastern DRC were sent to the UVRI VHF laboratory by Médecins Sans Frontières Switzerland staff working in that region. The initial diagnostics and subsequent full genome sequence analysis confirmed the presence of BDBV in the clinical samples from the Isiro health zone, but not from Dungu. The analysis showed that the viral genomes present in the Isiro clinical samples were ~98.6% identical to those of the original BDBV isolated in the Bundibugyo District of western Uganda in 2007⁷⁹. A total of 36 cases were confirmed by the end of the outbreak (officially declared on November 26th, 2012).

In October 2012, samples were received by the UVRI VHF laboratory from VHF alert cases in the Kabale and Ibanda Districts in southwestern Uganda. Initial testing at UVRI identified the etiological agent as MARV. During the next 3 weeks, a total of 15 additional cases were confirmed in the districts of Kabale, Ibanda, Mbarara, and Kampala based on testing at the CDC mobile laboratory in Kabale and/or serology at the VHF laboratory at UVRI. Preliminary epidemiological investigations identified 2 chains of transmission: 1) a patient who became infected in Ibanda and spread the virus to persons in Kabale, some of whom sought care in Kampala; and 2) patients in Ibanda, some of whom sought care in Mbarara. No epidemiologic data could be found to link these 2 clusters, but sequence analysis of complete viral genomes in serum samples from 1 acute case from each cluster showed nearly identical sequences (~99.9%), indicating that these cases were likely part of the same human-to-human transmission chain. Moreover, these viral genome sequences were highly similar (~99.3%) to 2 MARV isolates previously found in *R. aegyptiacus* bats (Figure 17) captured in 2008 and 2009 in nearby Python Cave in Queen Elizabeth National Park, southwest Uganda¹⁰⁹. Uganda MOH officially declared the end of the outbreaks in Kabale and Ibanda Districts on December 21st, 2012.

In November 2012, the UVRI VHF laboratory received samples from VHF alert cases in Luweero District, subsequently confirming SUDV infection. Over the next few weeks, a total of 6 cases were confirmed from the relatively close districts of Luweero, Jinja, and Nakasongola. Only 5 days passed between the laboratory diagnosis of the first case and the last acute case. Nearly identical (~100%) viral NP gene sequences were found in the viruses detected in 3 serum samples, suggesting a single chain of human-to-human transmission. Moreover, the full-length genomic sequence obtained from a viral isolate showed closest identity to the SUDV Gulu strain isolated in Uganda in 2000¹⁰⁸, with the SUDV Kibaale isolates identified in 2012 was more genetically distant. The end of this outbreak was officially declared on January 16th, 2013.

3.6 Objective 6: Utilize multiple investigations and studies to characterize the re-emergence of RVFV in Uganda.

The final manuscripts focus on identifying, investigating, and characterizing RVFV in Uganda. In March 2016, the UVRI VHF surveillance program confirmed the first outbreak of RVFV in Uganda since 1968.

3.6.1 Objective 6 Part 1: Perform a comprehensive epidemiological investigation and phylogenetic analysis of the RVFV isolates to identify the source(s) of the outbreak (Manuscript 6)

In total, 4 laboratory-confirmed acute RVF cases and 2 probable deaths were identified in Kabale District. A multi-disciplinary team carried out a rapid and comprehensive outbreak investigation within 2 days of initial laboratory confirmation, including collecting human, livestock, and mosquito samples to identify additional cases and possible sources of infection. Laboratory samples collected from 19 family and community members of confirmed and probable cases revealed 2 additional seropositive, convalescent RVF patients, showing that RVFV has been circulating in Uganda prior to detection of this outbreak. In addition, 8 of the 83 (9.6%) livestock samples collected showed evidence of recent RVFV infection. A total of 6 different genera of mosquito vectors, representing 33 species and sub-species, were collected near the residences of confirmed and probable patients. RVFV was detected in 3 of 298 (1%) pools tested by RT-PCR, suggesting active vector transmission at the time of the outbreak.

3.6.1.1 Outbreak investigation

Following laboratory confirmation of the 2 initial cases, a multidisciplinary team from the Uganda MOH, UVRI, MAAIF, CDC-Uganda, WHO, and Kabale District local government conducted the initial investigation. The primary objectives were to conduct a detailed epidemiological case investigation, initiate enhanced surveillance and active case finding, establish a designated treatment unit at KRRH, and survey active animal morbidity and mortality. The epidemiology and surveillance team provided case definitions (suspected, probable, and confirmed) for RVF used to help identify new human cases in the community. A suspected case was anyone presenting with acute onset of fever ($>37.5^{\circ}\text{C}$), a negative malaria test, and at least 2 of the following 3 symptoms: headache, muscle or joint pain, and any gastroenteritis symptom (nausea, vomiting, abdominal pain, and diarrhea). A probable case was any unexplained death in the community, and any suspected case that had thrombocytopenia, low white blood cell counts, or raised hematocrit, plus at least one of the following: bleeding tendencies, sudden change in vision, or jaundice. A confirmed case was any suspected or probable case with a laboratory confirmation by either detection of RVFV nucleic acid by RT-PCR or demonstration of serum IgM antibodies by ELISA.

The laboratory team worked to improve rapid case detection by facilitating sample collection and transport, and fast-tracking RVFV testing of field and hospital specimens taken from humans and livestock. Field and hospital-based personnel were also trained in the proper use of personal protective equipment (PPE) while collecting, processing, and handling specimens. Additional investigation teams collected human, livestock, and vector samples from high-risk locations to identify further RVF cases; these locations included the main abattoirs, district health centers, and hospitals.

3.6.1.2 Investigation of confirmed and probable cases

The teams visited the homes of the 2 confirmed acute patients (AC1 and AC2, discussed below) to collect additional information about them and assess potential exposure and risk factors. The team interviewed family members and community residents in attempts to identify additional cases. Interviews using standard case investigation forms were conducted with all identified persons. Blood samples were collected from all suspected patients, as well as from family members with whom an interview had been conducted. In addition to the 2 acute cases, a suspected probable RVF death, probable case 1 (PC1), was reported to the district surveillance officer in Burorane Village, Kyanamira Subcounty, on March 9th. On March 11th, one additional report was received of a death suspected to be caused by RVFV in Mushenyi Village, Katuna, near the Rwandan border, and was listed as probable case 2 (PC2). Investigation teams were dispatched to obtain more detailed information and collect blood samples.

3.6.1.3 Community investigations

The investigation team collected blood samples from household members of confirmed and probable patients, as well as from neighbors in the affected villages, to identify any additional acute or convalescent RVFV cases. In total, 19 human blood samples were collected during the initial rapid investigation from various locations in Kabale District (Figure 18). All samples were tested for RVFV by RT-PCR and for anti-RVFV IgM and IgG by ELISA.

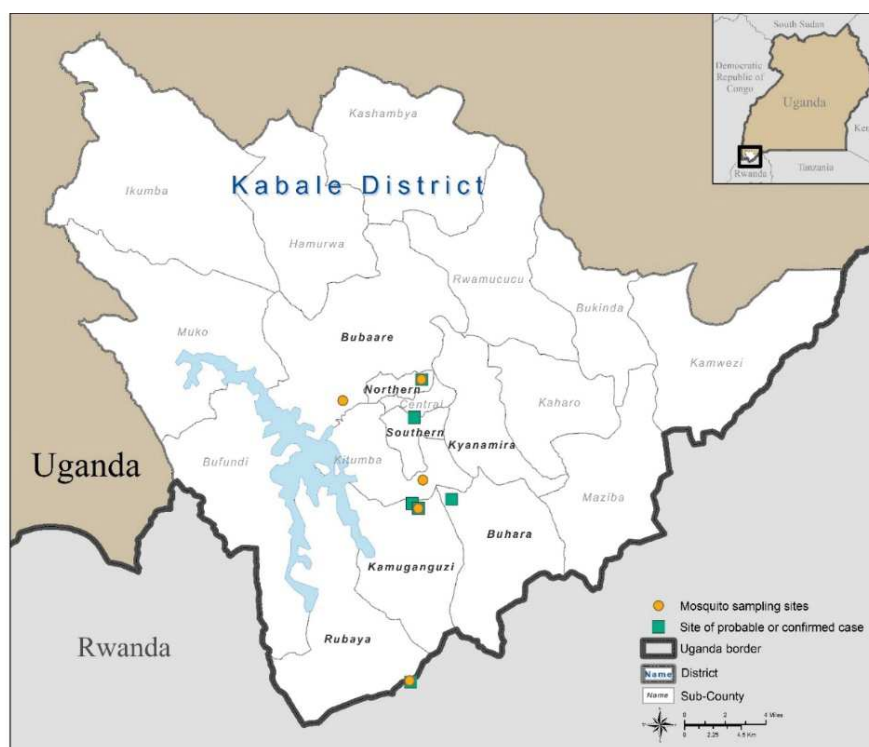


Figure 18: Map showing the locations of confirmed and probable RVFV cases and locations where human, livestock, and mosquito samples were collected during the outbreak investigations in Kabale District¹³

3.6.1.4 Livestock investigations

In addition to collecting human blood samples during the initial investigation, blood samples from domestic livestock in the same locations were also collected. Because RVF in animals may be clinically hard to define, blood samples were collected at or near the households where suspected, probable, or confirmed human cases had been identified (Figure 18). Blood samples were also collected from livestock at the central Kabale town abattoir. In total, 83 livestock samples were obtained from bovine, caprine, and ovine species. Whole blood EDTA samples were collected from the jugular veins using vacutainer EDTA collection tubes. Information about the clinical history of individual animals and the herd was also recorded. The samples were tested by both RT-PCR and IgG serology.

3.6.1.5 Entomological investigations

Mosquitoes were collected in 5 locations in Kabale district (Figure 18). Mosquitoes were trapped in Bugongi Village in Northern Division near the home of first confirmed case, and in Omururinda Village, Kamuganguzi Subcounty, near the home of the second confirmed case. They were also collected in Mushenyi Village, Rubaya Subcounty; in the home of a probable case in Kazigizigi Village, Kitumba Subcounty, located near Kabale town council; and in Nyakayenje Village in Bubaale Subcounty near Lake Bunyoni (Figure 18). Mosquitoes were identified using appropriate keys¹¹⁰⁻¹¹², processed for identification on a chill table mounted on a Zeiss Discovery V12 microscope, and pooled according to village, sex, and feeding status.

The initial acute case, AC1, was a 48-year male butcher from Upper Bugongi, Northern Division, Kabale municipality. On March 2nd, 2016, he reported an acute onset of a high-grade fever, severe headache, and joint pains. On March 3rd, he traveled to KRRH to seek treatment for suspected malaria, was discharged, and returned home. By March 9th, 7 days after onset of symptoms, he still had a fever and had developed other symptoms that included loss of appetite, generalized body weakness, severe anemia, ocular jaundice, and hemorrhagic symptoms (bleeding from the conjunctiva and nose, and bloody stool). He reported back to KRRH and was admitted for high risk of renal and liver failure. The attending physicians suspected VHF and obtained a blood sample to send to UVRI for testing. The sample was received at the UVRI VHF laboratory on March 10th, tested, and found positive for RVFV by RT-PCR ($C_T = 32$).

The second acute case, AC2, was a 16-year-old male student from Omururinda Village, Kitumba Parish, Kamuganguzi Subcounty. Records at KRRH showed that he first reported to the out-patient facility on February 10th, 2016, with a 10-day history of fever, headaches, and joint pain. On this visit, he tested negative for malaria by both microscopy and malaria rapid diagnostic test (mRDT). He was treated with cotrimoxazole (septrin) and mebendazole. On February 22nd, he presented to Kamukira Health Center IV in Kabale town reporting symptoms consistent with relapsing fever. He again sought care to manage the same fever as an out-patient at KRRH on February 25th, and was given anti-malarial drugs, treated symptomatically, and discharged. On March 10th, he presented at KRRH again with a high-grade fever (39°C) and hemorrhagic signs, including bloody sputum, nose bleeds, bloody urine, and bloody stool for 3

days, as well as a very tender abdomen. He had a low platelet count and was anemic (HB 7.9 g/dL), with high amylase levels but normal creatinine, and was treated for epistaxis with cotton nasal packs. He had tested negative for malaria by microscopy, for typhoid fever and hepatitis B by hepatitis B surface antigen (HbSAg), and for brucellosis by buffered *Brucella* antigen test (BAT). A blood sample was collected and sent to UVRI for testing suspected VHF. He was isolated the following day and given supportive therapy. By the time of his isolation, he had started convulsing and remained unconscious for 6 days. On March 11th, RVFV was detected in the sample by RT-PCR ($C_T = 30$).

Probable case 1 (PC1) was a 30-year-old male butcher from Burorane Village, Kyanamira Subcounty, Kabale District, who died on February 16th, 2016. The investigation team interviewed family members, who reported that his symptoms began on February 13th and included headache, fever, and diarrhea, followed by convulsions and mental disorientation. On February 14th, he developed hemorrhagic signs, including nose bleeds and vomiting blood, as well as hiccups and loss of consciousness. Bleeding was reported to continue following his death.

Probable RVF case 2 (PC2) was a 30-year-old male farmer from Mushenyi Village, Katuna, near the Rwandan border, who first felt ill on March 4th. He resided less than a kilometer from the residence of the grandparents of AC2 but was not otherwise epidemiologically linked to the grandparents or AC2. This patient experienced symptoms of headache, fever, muscle pains, bleeding from the mouth, mental disorientation, and loss of vision, and was taken to a clinic near the border, where he received IV fluids. He was later transferred to Rugarama hospital near Kabale town, where he died on March 10th and was buried on March 12th.

No additional acute human RVFV cases were identified by RT-PCR. Low levels of both IgM and IgG were detected in one sample collected from a 36-year-old male farmer residing in Mushenyi Village, approximately 25 km south of Kabale town (Table 1). He had no epidemiological links to AC1, AC2, PC1, or PC2. Mushenyi Village is approximately 3 km from Omururinda Village where the second confirmed case resided and was presumed to have been infected. The man reported onset of fever on March 3rd, but began to recover starting on March 10th. Other symptoms included intense fatigue, chest and muscle pains, and headache. On March 13th, a sample was collected from him during the initial outbreak investigation and sent to UVRI VHF laboratory. No RVFV was detectable by RT-PCR, and anti-RVFV IgM and IgG titers were 1:400. This case was classified as the third acute confirmed case, AC3.

In addition, one convalescent IgG-seropositive case, convalescent case 1 (CC1), was identified from Mushenyi Village: a 26-year-old male farmer. He reported some symptoms compatible with RVF, such as fever, headache, abdominal pain, fatigue, and chest pain, but did not seek medical care. Although he had contact with livestock, none of the animals were sick recently or at the time of sampling. A second convalescent IgG-seropositive case, CC2, was identified from Burorane Village, Kyanamira Subcounty. This patient was a carpenter and the brother of PC2,

described above. At the time of assessment, he complained of headache and cough, but no other recent symptoms compatible with RVFV infection. He reported no direct contact with animals or recent travel outside of Kabale District.

Investigation site (subcounty)	RVFV IgM seropositive	RVFV IgG seropositive	RT-PCR positive	Notes
Central Division				
Household #1				
Human (n = 0)	--	--	--	Home of AC1
Livestock (n = 5)	--	0 (0%)	0 (0%)	
Abattoir				
Livestock (n = 45)	--	2 (4.4%)	0 (0%)	Kabale central abattoir
Kamuganguzi				
Human (n = 5)	0 (0%)	0 (0%)	--	Home of AC2
Livestock (n = 12)	--	2 (16.7%)	1 (8.3%)	
Rubya				
Household #1				
Human (n = 5)	1 (20%)	2 (40%)	--	Home of PC1
Livestock (n = 10)	--	4 (40%)	0 (0%)	
Household #2				
Human (n = 4)	0 (0%)	0 (0%)	--	Home of grandparents of AC2
Livestock (n = 11)	--	0 (0%)	0 (0%)	
Kyanamira				
Human (n = 4)	0 (0%)	0 (0%)	--	Home of PC2
Livestock (n = 0)	--	--	--	

Table 1: Summary of human and livestock investigations, sampling, and testing, Kabale District, 2016. AC1, acute case 1; AC2, acute case 2; PC1, probable case 1; PC2, probable case 2.¹³

By March 27, 2016, after the initial outbreak investigations were concluded, 3 confirmed acute RVF cases (AC1, AC2, and AC3) had been identified, and 19 more suspected cases were investigated; all suspected cases were negative for RVFV. Two months later, on June 7th, a 35-year-old male mason who resided in Rushaki Village, Southern Division, Kabale District, presented to and was admitted at KRRH with fever and symptom that began on June 2nd. Symptoms included vomiting, intense fatigue, abdominal pain, chest and muscle pains, headache, cough, conjunctivitis, sensitivity to light, and hemorrhage. A sample was collected upon admission and sent to UVRI for testing; it was found to be RVFV-positive by RT-PCR ($C_T = 21.3$) on July 9th and the patient was confirmed as the 4th acute case 4 (AC4). The patient continued to receive supportive care at KRRH, and subsequent blood samples were drawn to monitor RVFV infection and serological response.

Following confirmation of acute RVFV in AC 1 and AC2, each patient was placed into isolation and provided supportive treatment. Successive blood samples were collected from each patient during treatment to monitor RVFV RNA levels in the blood by RT-PCR and the progression of the patients' immune response through IgM and IgG serology is shown in Figure 19.

On admission (8 days after symptom onset), AC1 had an RT-PCR C_T value of 32 and an IgM titer of $\geq 1:6400$, indicating acute infection. Subsequent blood samples were obtained over the course of the patient's isolation 12, 16, 22, and 26 days post symptom onset. The viral C_T value first increased slightly ($C_T = 34.7$ on day 12), but remained relatively stable, with C_T values of 33.5, 33.5, and 35.15 on days 16, 22, and 26, respectively. IgM titers remained constant at $\geq 1:6400$ at all time points tested. IgG titers were undetectable throughout the course of isolation, except at day 26 post symptom onset, when IgG titers of 1:400 was found. No additional samples were taken and the patient was recommended for discharge on April 26th, 2016.

AC2 was admitted into isolation at KRRH 15 days after symptom onset, with an RT-PCR C_T value of 30 and an IgM titer of $\geq 1:6400$, indicating acute infection. Subsequent blood samples were taken 19, 26, 31, and 41 days post symptom onset. The viral C_T value decreased slightly at 19 days post onset to 28.7, increased on days 26 and 31 to 30.4 and 31.4 respectively, and then decreased slightly to 29.39 by day 41. As with AC1, IgM titers remained constant at $\geq 1:6400$, and no IgG was detected until day 41 (1:1600 titer). No additional samples were taken and the patient was recommended for discharge on April 26th, 2016. He remained in isolation for supportive treatment after developing some ocular complications as a result of RVFV infection.

AC3 was identified during the initial epidemiological investigations in the community, and thus only one sample was collected from this individual, on March 13th.

AC4 was admitted into isolation at KRRH on June 7th, 7 days post symptom onset, with initial RT-PCR C_t value of 21.3 and no detectible IgM or IgG titers. Subsequent blood samples were collected 11, 20, and 28 days post symptom onset. The viral C_T value increased slightly to 23.8 at 11 days post onset, and then increased more dramatically to 32.2 and 32.1 on days 20 and 28, respectively. IgM was detectable on day 20, rising dramatically to $\geq 1:6400$ titer; this titer then remained constant. IgG titer was first detected on day 20 (1:6400 titer); this also remained constant. No additional samples were collected, and the patient was recommended for discharge.

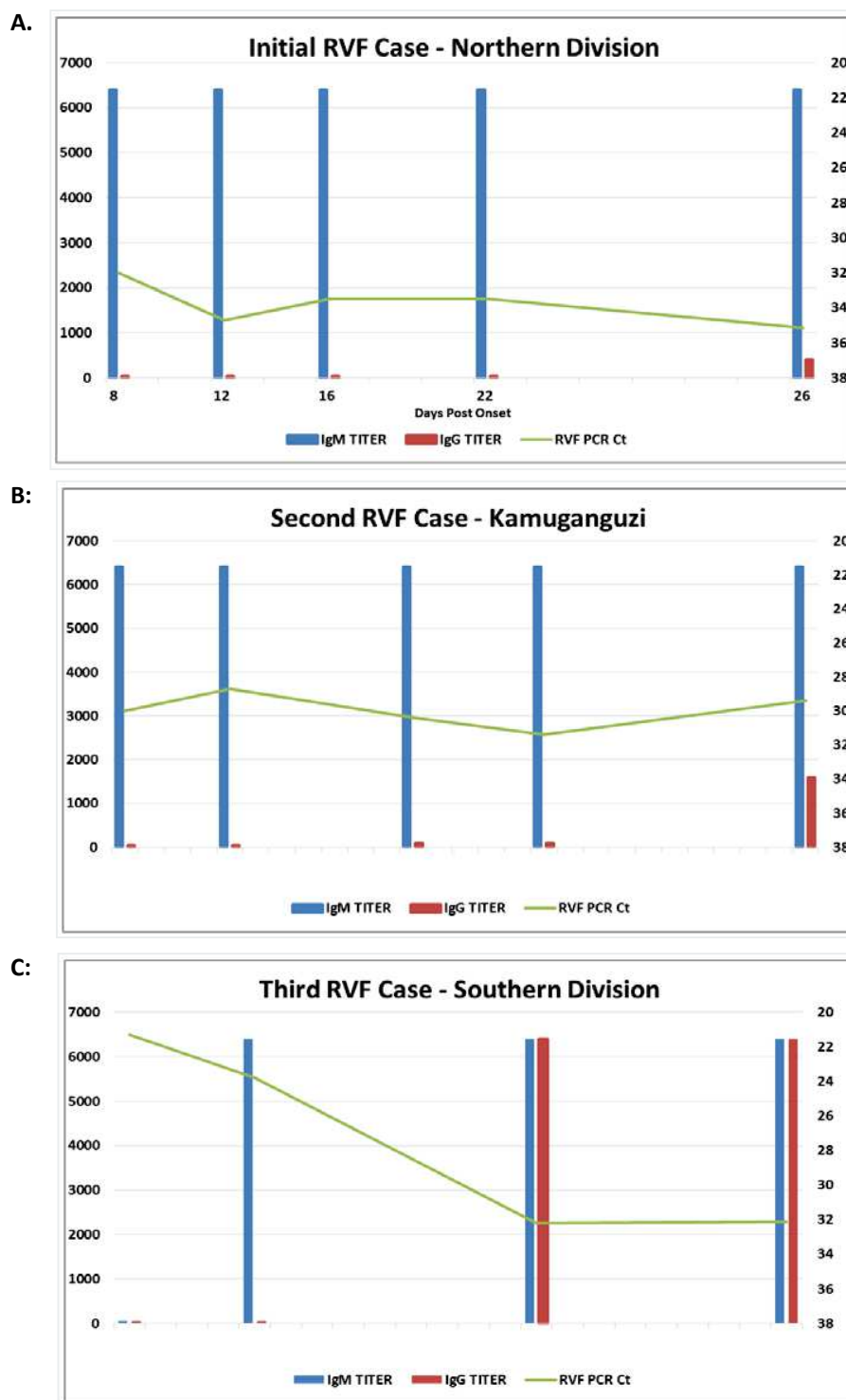


Figure 19: Sequential RVFV IgM and IgG serology and RT-PCR results for the 3 cases in Kabale District confirmed to be acute clinical RVF cases AC1 (A), AC2 (B), and AC4 (C). X-axis represents days after RVF symptom onset when blood sample was collected; y-axis (left) represents IgG serological titers; y-axis (right) represents cycle threshold (C_T) values for RT-PCR performed on each sequential clinical sample.¹³

In total, 8 of 83 (9.6%) livestock were seropositive for RVFV by IgG ELISA (Table 1). Only one animal, a goat from Omururinda Village where AC2 resided, was also positive by RT-PCR ($C_T = 33.3$), showing acute infection. IgG serology confirmed the goat to have been infected (titer $\geq 1:6400$). An interview with the animal owner revealed that the goat had a previous abortion with the death of one kid in February 2016.

The vector investigations resulted in a total of 298 pools, representing 9950 mosquitoes, collected from the 5 locations listed above. The predominate pooled species sorted for RT-PCR testing was *Aedes gibbinsi* (24.2%) followed by *Coquillettidia fuscopennata* (13.4%) and *Aedes tricholabis* (11.4%). In total, 6 genera and 33 species of mosquitoes were identified. Only 3 (1%) mosquito pools were found positive for RVFV by RT-PCR. One positive pool was *A. gibbinsi* (1.4%) trapped in Mushenyi Village, home of PC1. The second positive pool was unspecified *Aedes spp.* (12.5%) trapped in Kazigizigi, Southern Division, Kabale town. The third positive pool was *C. fuscopennata* (2.5%) trapped near the home of AC2.

Clinical samples were sent to CDC in Atlanta, USA, for further processing and analysis. At the CDC, virus isolation was attempted using all blood samples serially collected from the 3 RT-PCR positive acute RVF cases, AC1, AC2, and AC4. Only the first acute sample from each patient yielded RVFV. The viral isolates obtained from these initial samples were used for genetic sequencing. Complete S, M, and L segment genome sequences from RVFV isolated from AC1, AC2, and AC4 were obtained. All 3 segments were the most closely related to the previously identified RVFV isolated in Kenya in 2007 and Sudan in 2010 (Figure 20). There was little difference in the overall nucleotide lengths of the S, M, and L segments of the 3 Kabale isolates compared to the Kenya-2007 and Sudan-2010 sequences: S segment was 1,690–1,691 nt, M segment was 3,885 nt, and L segment was 6,404 nt. Pairwise nucleotide identity between the 3 Kabale RVFV isolates ranged from 94.7–98.4% for S segment, 99.3–99.8% for M segment, and 99.2–99.9% for L segment. Amino acid identity between the 3 isolates was 100% for S segment, 99.2–100% for M segment, and 99.8–99.9% for L segment.

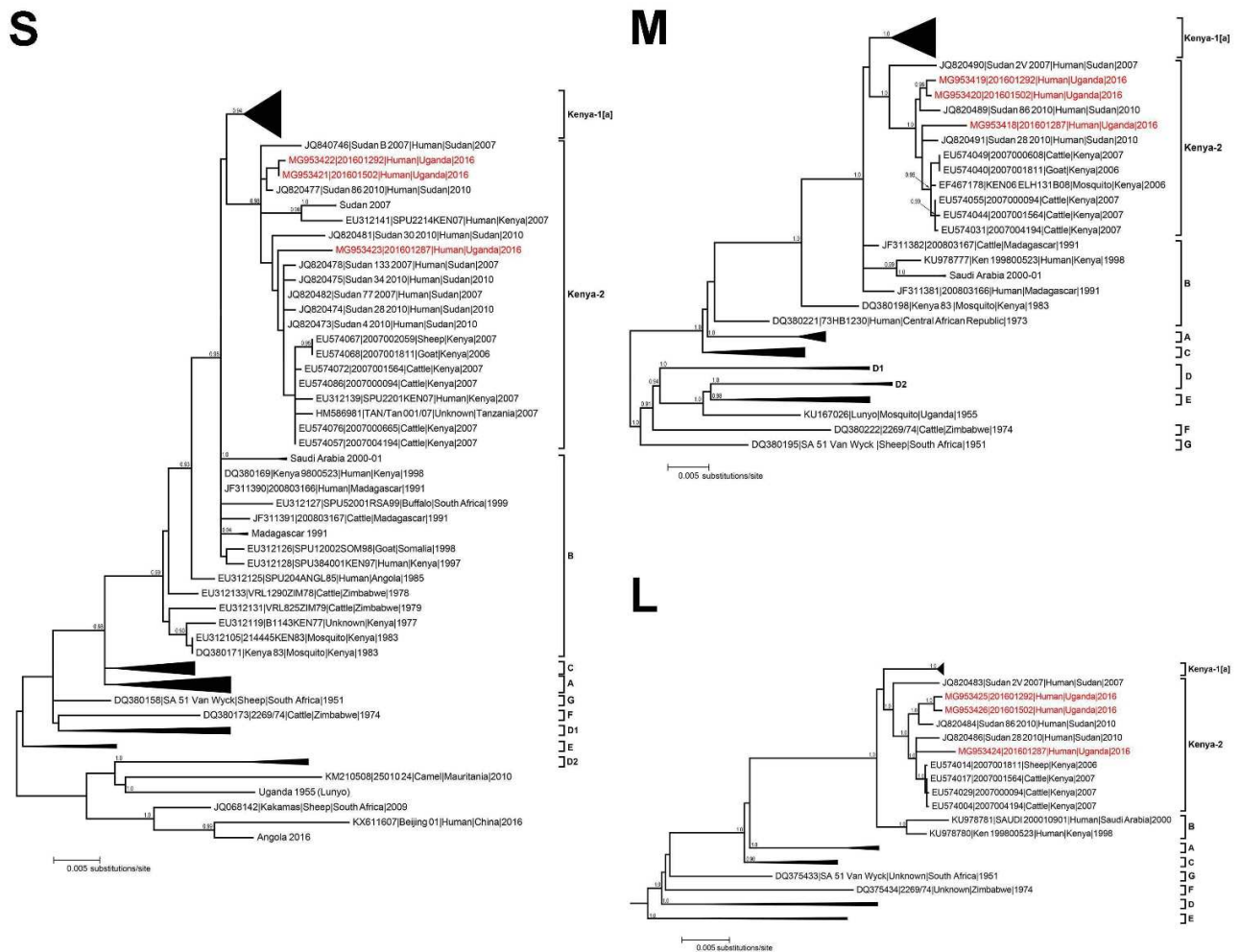


Figure 20: Phylogenetic trees comparing complete S, M, and L segment sequences of RVFV using all available full genome sequences. The sequence from the three RT-PCR–positive acute human cases described here, 20160187 (AC1), 201601298 (AC2), and 201601502 (AC4), are in red type. The evolutionary history was inferred based on the SPR model with the GTR + Γ ($n = 4$) nucleotide substitution model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Branch support estimates represent bootstrap values following 1,000 replicates and are displayed as integers for branch support > 70%.¹³

Maximum-likelihood phylogenetic analyses of the 3 Kabale RVFV isolates showed them all clustering in the same positions regardless of segment analyzed. Sequences from the first acute Kabale case, AC1 (201601287), grouped in the Kenya-2 clade. All sequences were closely related to human sequences identified in Sudan in 2007 and 2010, and in Kenya in 2007. Viruses within the Kenya-2 clade have been previously associated with the large epizootic occurring in East Africa in 2006–2007⁷⁴. The Sudan 2010 lineage was associated with human cases identified as part of enhanced surveillance efforts in Sudan following an epizootic that occurred in several Sudanese states in 2006–2007 and was linked with the larger East African epizootic occurring during the same time period⁶⁸. No genetic evidence of multiple RVFV lineages circulating in Kabale District was seen, nor was any evidence of virus re-assortment within the samples analyzed. Even though these sequences were obtained from geographically distinct locations within Kabale District, sequence analysis showed that these isolates belonged to a singular lineage and suggested at least 2

introductions of RVFV into Kabale District. Furthermore, these data suggest that the progenitor viruses of the 2016 Kabale RVFV outbreak was likely related to RVFV that was responsible for the outbreak in East Africa in 2006–2007.

3.6.2 Objective 6 Part 2: Determine the seroprevalence of RVFV in humans and domestic livestock species to identify risk factors for RVFV infection in select areas in Uganda (Manuscript 7)

Following this initial RVFV outbreak detection, a study to assess the seroprevalence of RVFV in humans and animals living in and around Kabale District was initiated. The objectives of the study were to determine the seroprevalence of RVFV in both humans and animals in Kabale and surrounding districts, identify risk factors and high-risk areas for RVFV, determine if RVFV is emerging or endemic, and identify unrecognized RVF cases that may be related to the 2016 outbreak. Overall, evidence of RVFV seropositivity in 13% of humans and animals sampled was found. The study also showed that butchers and persons who handled raw meat were most likely to be RVFV seropositive. Although no RVF cases had been detected in Uganda from 1968 to March 2016, the study suggests that RVFV has been circulating undetected in both humans and animals living in and around Kabale District. RVFV seropositivity in humans was associated with occupation, suggesting that the primary mode of RVFV transmission to humans in Kabale District could be through contact with animal blood or body fluids.

From April 1–12, 2016, 34 locations in and near Kabale District were selected for inclusion in the serosurvey by a multidisciplinary team consisting of individuals from Kabale District, Uganda MOH, Uganda MAIF, UVRI, and CDC (Figure 21).

segment. Briefly, following heat and detergent inactivation, specimens were tested by anti-RVFP-specific IgM and IgG ELISA using inactivated RVFP-infected Vero-E6 cell antigens and 4 dilutions of each specimen (1:100, 1:400, 1:1600, and 1:6400). Titers and the cumulative SUM_{OD} minus the background absorbance of uninfected control antigen (adjusted SUM_{OD}) were recorded. Samples were deemed positive if both the adjusted SUM_{OD} and the titer were above pre-established conservative cutoff values of ≥ 0.45 for IgM ELISA and ≥ 0.95 for IgG ELISA⁹⁵.

A total of 655 persons participated in the serosurvey. Participants were recruited at the Kabale town abattoir (n = 117; 18% of participants), from villages where a recent acute RVFP case had been identified (n = 237; 37%), and from villages with no recorded outbreaks (n = 293; 45%). Most participants (n = 396; 60%) were aged 20–49 years and had completed primary education (n = 360; 55%). The most common occupation listed was farmer or herdsman (n = 335; 52%), and most individuals owned animals (60%). Contact with animals was common, with 78% (n = 508) of participants reporting contact with animals in the past year. A total of 1,051 animals were sampled. Of these, 324 (31%) were cattle, 569 (54%) were goats, and 157 (15%) were sheep. Most were adults (n = 620; 59%) and a local breed (n = 829; 79%).

Of all persons tested, 13% (88/655) were RVFP seropositive. Three (0.5%) persons had anti-RVFP IgM only, 78 (12%) had IgG only, and 7 (1%) had both IgM and IgG. Two individuals positive for RVFP IgM also tested positive for RVFP RNA by RT-PCR, suggesting active infection at the time of sampling. The 3 IgM-only positive individuals (one trader, one housewife and one farmer) were all from the village in which one of the initial acute human RVF cases was living, but were not related to that individual. No persons under 20 years of age were RVFP seropositive, while 17% (n = 66) of individuals aged 20–49 years were seropositive. Of individuals 50 years and older, 11% (n = 22) were seropositive. Butchers were the most likely to be RVFP seropositive, with 35% showing evidence of seropositivity. Other occupations evaluated for RVFP seropositive included farming at 10%, housekeeping at 8% (4/49), teaching at 18% (2/11) and trading at 12.5% (3/24). In the bivariate analysis, increasing age ($\chi^2 = 14.4$; $p = 0.001$), male sex ($\chi^2 = 11.9$; $p = 0.001$), occupation as a butcher ($\chi^2 = 54.7$; $p < 0.001$), history of slaughtering or butchering animals ($\chi^2 = 23$; $p < 0.001$), and preparing raw meat ($\chi^2 = 13$; $p < 0.001$) were all significantly associated with an increased risk of RVFP seropositivity.

Multivariate logistic regression confirmed that being a butcher and handling raw meat was significantly associated with RVFP seropositivity, with an adjusted OR of 5.1 (95% CI 1.7–15.1; $p = 0.003$) and 3.4 (95% CI 1.2–9.8; $p = 0.024$), respectively (Table 2). Age, sex, slaughtering/butchering, and contact with animals through grazing were not significantly associated with RVFP seropositivity in the multivariate model.

The association between animal seropositivity and human seropositivity within a subcounty was examined using multivariate logistic regression, adjusting for contact with raw meat and occupation because these were found to be

significant risk factors in the univariate analysis. Human seropositivity within a subcounty was found to be associated with animal seropositivity, with OR of 1.1 (95% CI 1.0–1.1; $p < 0.001$).

Table 2: Multivariate analysis of risk factors for RVFV seropositivity in humans¹⁴

Variable	Seronegative	Seropositive	Adjusted odds ratio (OR)*	P value	95% CI
Sex					
Female	203 (93%)	15 (7%)	Ref		
Male	355 (83%)	71 (17%)	2.3	0.073	0.92–5.7
Age (as a continuous variable)				0.054	
Occupation					
Other	186 (93%)	15 (7%)	Ref		
Herdsman/farmer	302 (90%)	33 (10%)	1.1	0.78	0.47–2.7
Butcher	75 (65%)	40 (35%)	5.1	0.003	1.7–15.1
Live animal contact					
Grazing	319 (91%)	33 (9%)	0.84	0.62	0.41–1.7
Meat preparation					
Slaughtering/butchering	185 (78%)	53 (22%)	0.67	0.40	0.27–1.7
Handling raw meat	362 (84%)	71 (16%)	3.4	0.024	1.2–9.8

*Adjusting for age as a continuous variable in addition to the variables listed in the table

Alongside this serosurvey, a KAP study was conducted in Kabale District, and the findings of this KAP study were used to design health education materials targeting different stakeholders. For example, the educational materials targeting farmers and butchers emphasize reporting any sick animals to veterinarians, washing hands after touching raw meat or milk, cooking meat and milk thoroughly, using mosquito bed nets, and wearing more protective clothing when working in high-risk areas.

3.6.3 Objective 6 Part 3: Describe the knowledge, attitudes, and practices associated with RVF in Uganda (Manuscript 8)

Following the diagnosis of RVF cases in March 2016 in southern Kabale District, a KAP survey to identify knowledge gaps and at-risk behaviors related to RVF was conducted. A multidisciplinary team interviewed 657 community members, including abattoir workers, in and around Kabale District. Most participants (90%) had knowledge of RVF and most (77%) cited radio as their primary information source. Greater proportions of farmers (68%), herdsmen (79%), and butchers (88%) thought they were at risk of contracting RVFV compared to persons in other occupations

(60%, $p < 0.01$). More farmers and butchers (36% and 51%, respectively) had knowledge of RVF symptoms in animals compared to those in other occupations (30%, $p < 0.01$). Overall, knowledge, attitudes, and practice regarding RVFV in Kabale District could be improved through educational efforts targeting specific populations.

A total of 657 participants were interviewed; most (40%) were males aged 20–49 years. The mean age of participants was 40 years, with a range of 7 years to 90 years. Most participants were from sites where no RVF cases had been identified (293/647; 45%), but 238 (37%) were recruited from sites where a previous RVF case had been identified, and 117 (18%) of participants worked at the main abattoir in Kabale. Most participants owned domestic animals and had contact with goats (353/504; 70%), cattle (299/505; 59%), pigs (129/505; 26%), sheep (98/506; 19%), and poultry (91/505; 18%). Participants who owned livestock had, on average, 3 cattle, 4 goats, and 4 sheep. Few participants had contact with ducks (3/506; 0.6%) or rabbits (9/506; 2%).

Participants most frequently had contact with live animals during grazing (Table 3). A third (33%) of participants also reported assisting with animal births. Contact with dead animals was mainly through handling raw meat (77%). However, nearly half of the participants said that they were involved in slaughtering or butchering. Significantly more men than women were engaged in milking, birth assistance, and slaughtering/butchering (Table 2). However, significantly more women assisted with animal grazing. Few participants drank raw milk or ate raw meat.

Table 3: Types of contact with animals and animal products by persons participating in the RVFV KAP survey¹⁵

Type of animal contact	Female n (%)	Male n (%)	P value*	Total n (%)**
Live animal contact (n = 506)				
Milking	11 (7.8%)	60 (17%)	< 0.01	72 (14%)
Grazing	128 (91%)	218 (61%)	< 0.01	354 (70%)
Grooming	45 (32%)	82 (23%)	0.04	131 (26%)
Caring for sick animals	25 (18%)	88 (25%)	0.09	113 (22%)
Assisting with birth	24 (11%)	182 (44%)	< 0.01	211 (33%)
Sleeping near animals	18 (13%)	44 (12%)	0.9	64 (13%)
Dead animal contact				
Slaughtering/Butchering (respondents = 519)	17 (12%)	219 (60%)	< 0.01	238 (46%)
Handling raw meat (respondents = 561)	143 (83%)	284 (75%)	0.03	444 (77%)
Eating practices				
Drinking raw milk	5 (2.3%)	31 (7.3%)	< 0.01	36 (6%)
Eating raw meat	6 (2.8%)	24 (5.6%)	< 0.01	30 (5%)

*Comparing males to females using chi-square test.

**Total column includes respondents who did not specify gender.

Most (90%) of participants said that they had heard of RVF previously; a greater proportion of butchers (95%), herdsmen (94%), and farmers (92%) had heard of RVF compared to persons in other occupations (85%) ($\chi^2 = 10.4$; $p = 0.016$). Most respondents had heard of RVF from the radio (77%).

Interviewers asked participants if they had knowledge of RVF symptoms in humans and animals, and participants responded with yes, no, or do not know. Most participants, regardless of their occupation, said that they could identify RVF symptoms in humans; the most common human symptom cited by participants was bleeding. Less than half of participants listed fever, vomiting, or diarrhea as RVF symptoms in humans. Of all participants, 241 (37%) had knowledge of RVF symptoms in animals. Knowledge of RVF signs in animals was significantly higher among herdsmen and butchers than in other occupation groups. Bleeding was the most common RVF animal sign mentioned by participants, particularly butchers, farmers, and individuals in other occupations. Herdsmen were significantly more likely to identify fever as a sign of RVF in animals. In contrast, butchers were more likely to identify nasal discharge as an RVF sign in animals. In general, knowledge of RVF symptoms did not vary significantly by gender, but more women than men cited bleeding as a sign of RVF in humans. Significantly more men stated that they were familiar with RVF signs in animals, and significantly more men identified nasal discharge as an RVF sign in animals.

Of all participants, 53% (345/655) said that they had any knowledge about how RVFV is transmitted to humans or animals. Participants most frequently identified animal contact as a mode of transmission (269/348; 77%).

Participants were asked if RVFV could be transmitted by human-to-human physical contact (e.g., shaking hands) and through contact with human bodily fluids (e.g., blood). Of all participants who responded, 83 of 348 (24%) said RVFV could be transmitted through mosquitoes and through contact with body fluids of an infected person (84/348). Of farmers, 76 of 115 (66%) identified animals as a source of transmission, compared to 80–93% of butchers, herdsmen, and persons in other occupations ($p = 0.003$). Some participants thought that RVFV could be transmitted by human-to-human bodily contact (107/348; 31%) and through the air (25/348; 7%). The belief that RVFV could be transmitted by casual contact was most common among farmers (40%), compared to butchers (20%), herdsmen (20%), and others (29%) ($p = 0.021$). Most participants identified goats (267/288; 93%), cattle (260/290; 90%), and sheep (203/289; 70%) as sources of RVFV transmission. Although the survey did not specifically ask whether or not eating raw meat or drinking raw milk were risk factors, 38 participants mentioned these as other possible sources of RVF transmission.

The questionnaire also asked about ways to prevent RVFV transmission; most participants cited avoiding animals (257/612; 42%) and sick people (122/612; 20%) as modes of prevention. A small proportion of respondents (92/612, 15%) cited sleeping under a mosquito net as a method of prevention. Although the questionnaire did not specifically ask about boiling milk and cooking meat, participants frequently mentioned them as possible methods to prevent RVFV transmission.

Mosquito nets were used by most participants (82%; 539/655). Of those 116 individuals who did not use a mosquito net, the most frequently cited reason (given by 17% of these respondents) was that the nets were uncomfortable. Of

all participants, 29% (149/510) said that they used PPE (e.g. gloves, mask, gumboots) when handling animals. Participant responses regarding the use of PPE varied by occupation and gender. Butchers used PPE significantly more often than persons in other professions, but they mostly used gumboots and aprons, rarely gloves.

Most participants (88%; 501/567) believed that RVFV exists; this finding did not vary by gender but did vary significantly by occupation. Compared to herdsmen (90%) and farmers (95%), fewer butchers (83%) and persons in other occupations (85%) ($p < 0.05$) believed in the existence of RVFV. The most common reason for not believing that RVFV exists was that people were not aware of an RVF case near Kabale (23%; 36/156); just 28% (177/631) of all individuals were aware of local RVF cases. Significantly more males (30%) than females (22%) had heard of an RVF case ($p < 0.05$). Most people believed that they are at risk of getting RVF (69%; 448/651). Significantly more butchers identified themselves as being at risk of RVF (88%) compared to persons in other occupations (60%), farmers (68%), and herdsmen (79%) ($p < 0.01$). Similarly, a greater proportion of males (74%) identified themselves as being at risk of contracting RVFV compared to females (59%) ($p < 0.05$). When asked about RVF treatment, most individuals said that they would seek care from modern medicine (91%; 579/636). Most participants said that they would interact with RVF survivors (75%; 461/613) and welcome them back into their community (73%; 478/652).

4 Discussion:

The manuscripts described above make up the body of a scientific and epidemiological work and provide a comprehensive account of how initial molecular and genetic analysis tools first used for VHF outbreak detection can be further developed and then successfully utilized and implemented in real-world surveillance, investigations, and response.

Using a combination of RVFV IgM and antigen-capture ELISA tests, along with the RT-PCR assay, RVFV was quickly identified as the cause of a large outbreak in Saudi Arabia reported in September 2000. RT-PCR proved to be an excellent complement to the antigen and antibody ELISA detection systems for the initial rapid diagnosis of RVF. Virus-specific antibodies were present in 3 of the 4 specimens that were positive by both virus isolation and PCR but negative by antigen capture, suggesting that immune complex formation (antibody blocking of antigen) may be the basis for the lower sensitivity of the antigen-capture assay. Although the IgM assay failed to identify 9 of 17 laboratory-confirmed (by virus isolation or RT-PCR) acute RVF cases, the assay did detect recent RVFV infections in 5 contacts (mostly close family members) and one acute case that would have been missed on the basis of virus isolation or PCR alone. The data from this study and others demonstrate the importance of combining various assays for virus detection.

Phylogenetic comparison of the nucleotide sequence differences between the S, M, and L segments of the Arabian Peninsula RVFV isolate and those of previously characterized RVFV isolates showed a close relationship between the Saudi Arabia/Yemen RVFV and those circulating earlier in East Africa, particularly with the viruses responsible for the large RVF outbreak seen in the region in 1997–1998. These results suggest that RVFV was introduced into Saudi Arabia and Yemen from East Africa.

This RVF outbreak, the first confirmed outside Africa, illustrates the potential for this disease to spread to other regions of the world. RVFV activity on the Arabian Peninsula resulted in a considerable amount of illness from September 2000 to February 2001. The finding of 6 laboratory-confirmed RVFV infections among household contacts is consistent with the view that hospitalized patients represent only a small fraction of the number of infected persons. Based on these and earlier observations, the number of human infections during this epidemic must have been considerable.

After this RVF outbreak detection in Saudi Arabia, there was clear need to develop more accurate qRT-PCR-based diagnostic techniques to allow further characterization of this outbreak and provide a platform to investigate and characterize future outbreaks. The central feature of real-time PCR-based diagnostic techniques is the high analytical sensitivity and specificity afforded by unique primer and probe sites located on genomic nucleic acid templates. Unfortunately, many important human and veterinary pathogens have significant nucleotide variation across their known strain genomes, rendering the design of broadly reactive and highly sensitive primers and probes problematic. Analyzing the complete genome sequences of 40 biologically and ecologically diverse RVFV strains demonstrated that the overall nucleotide diversity of RVFV is low as the result of recent common ancestry.⁷⁴ This information provided a foundation to establish a rapid, high-throughput technique for qRT-PCR detection of all known RVFV strains.

The utility of this assay was tested by using human patient sera collected during the 2000 Saudi Arabia RVF outbreak. As expected, the mean overall patient RVFV RNA load was significantly lower in surviving patients than in those with fatal outcomes. Interestingly, even though the RVFV RNA load was initially high in some non-fatal patients, mean RNA loads decreased significantly between days 1 to 4 and days 5 to 8 after symptom onset. These data suggest that testing serial patient specimens collected 24–48 h apart may have prognostic utility in determining the progression of RVF disease and patient outcome.

The window for detecting RVFV by specific antigen-capture ELISA may be limited to be approximately 1 to 7 days after the onset of clinical symptoms in human patients. Due to its higher detection capability, qRT-PCR may continue to detect RVFV at least 10 days after the onset of symptoms. However, the above data show that the diagnostic value of qRT-PCR decreases significantly after 10 days post symptom onset. These findings taken together highlight the complementary nature of molecular detection assays and serologic tests and the importance of using a combination

of assays for reliable diagnosis of virus infection. Epidemiologic investigations of outbreaks occurring in regions with endemic RVFV activity are often complicated by the fact that some percentage of individuals will have had prior exposure to RVFV. Using both antibody and virus detection assays can therefore provide insights into disease status by differentiating between an enzootic and an epizootic region or between acute- and convalescent-phase individuals. For instance, detection of RVFV-specific IgG without concurrent detection of specific IgM antibody or acute viremia (by antigen capture or qRT-PCR) strongly suggests that the infection occurred at some previous time and is not the result of recent virus exposure. During outbreak investigations, information regarding the time of sample collection after illness onset is often unavailable to the testing laboratory. The lack of this information and of antibody testing may result in false-negative reporting of late acute-stage and convalescent individuals or animals. Thus, qRT-PCR assays are best used along with serological methods during outbreak investigations.

Comparing data from fatal and non-fatal cases revealed a striking difference in the levels of RVFV detected in the blood. Mean RVFV RNA levels were up to 10 C_T values (~3 log₁₀ PFU equivalents/mL) higher in initial samples from fatal cases than in non-fatal ones. In addition, viral RNA and antigen levels did not decline much prior to the death of these patients, presumably due to continued high replication of the virus in the virtual absence of detectable anti-RVFV IgM or IgG antibodies (Figure 20). Further studies will be necessary, but these findings based on a limited number of patients suggest that measuring initial RVFV loads may help identify high-risk patients, especially if follow-up samples can also be obtained. Currently, host-specific risk factors for RVF mortality are not understood, and early indicators like viral load may shed light on this important topic. Given that outbreaks frequently occur in rural areas of Africa and affect very large numbers of individuals (the Egyptian outbreak in 1977 to 1978 was estimated to have involved >200,000 human infections), rapidly identifying high-risk patients can have practical implications for patient management in these resource-limited settings.

The utility of the qRT-PCR assay was illustrated further when it was successfully used under field conditions in the diagnostic testing of more than 1,000 specimens from acutely infected livestock herds during the recent large RVF epidemic/epizootic in eastern Africa (Kenya, Somalia, and Tanzania) in late 2006 and early 2007. This validated and field-tested assay should have great utility in the diagnosis of acute cases during outbreaks of infection among humans and animals during naturally occurring outbreaks or after intentional release of the virus into previously unaffected areas. The molecular diagnostic developed is now considered the gold standard RT-PCR molecular diagnostic for RVFV.

Beginning in 2011, the VSPB of CDC-Uganda, in collaboration with UVRI and the Uganda MOH, established a first of its kind national VHF surveillance and laboratory program. The program allowed rapid identification of several high-profile VHF outbreaks. Uganda's VHF surveillance system has contributed to a dramatic improvement in time-to-response for VHF outbreaks. Earlier case detection and laboratory confirmation have led to faster public health

responses; isolating patients using infection control precautions^{10, 32, 33} has decreased secondary transmission in communities, and ultimately led to shorter and less severe outbreaks. Large outbreaks of SUDV and BDBV occurred in 2000^{78,79} and 2007²⁰, respectively, during a time of limited surveillance and outbreak detection capacity. After the implementation of this enhanced surveillance system, however, EVD was identified in a 12 year-old girl from Luweero District, representing the first time an EVD index case was confirmed in a laboratory in Uganda, and only the second documented occurrence of an identified single-case EVD outbreak¹¹³.

Rapid laboratory identification in this outbreak allowed mobilization of an investigation team one day after initial laboratory detection and the rapid establishment of an isolation facility at Bombo Military Hospital. In this instance, the initial high clinical suspicion of EVD by astute clinical staff, the appropriate use of PPE and barrier protection by hospital staff, and the rapid laboratory confirmation of EVD in-country likely contributed to limiting the size of this outbreak.

The timeliness of diagnostic confirmation and outbreak response had substantially improved from previous EVD outbreaks in Uganda (timeline shown in Figure 22), in which EBOV transmission occurred for multiple months before the outbreaks were detected^{20, 114, 115}. This improved response is mainly due to the establishment of a permanent high-containment laboratory at UVRI capable of performing diagnostic testing for filoviruses and other VHF in

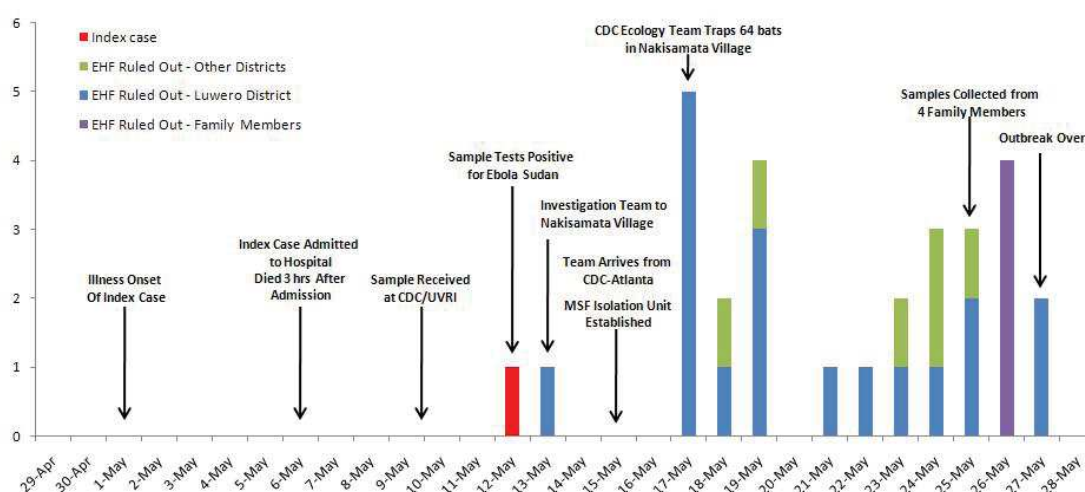


Figure 22: Timeline of SUDV outbreak, Uganda, 2011, showing key events in the investigation and response¹⁰

Uganda. The limited extent of this outbreak also demonstrates the powerful utility of a national VHF surveillance system coupled with the ability to rapidly diagnose VHF cases and respond to limit the spread of such high-hazard infections in the community or healthcare facility settings.

Since the detection of this initial EVD case in 2011, the number of subsequent outbreaks detected in Uganda through 2019 has now increased tenfold from the previous 10 years (Figure 23). However, the size and scope of each outbreak was dramatically reduced compared to previous outbreaks, as the time between initial clinical notification and laboratory confirmation had shortened from 2 weeks prior to 2010 to the current average of just 2.5 days. This speed has also directly led to rapid response and containment of these outbreaks and demonstrates the significant public health impact of having established, ongoing, and dedicated VHF surveillance to mitigate morbidity and mortality of endemic zoonotic infectious diseases. Moreover, Uganda's VHF program provides direct technical assistance and support for VHF outbreak responses throughout Africa, including the West Africa EVD outbreak of 2013–2016¹¹⁶. Program materials developed for the Uganda VHF program, including standardized case reporting forms, case definitions, health education and risk communication materials, and the Epi-Info VHF application¹¹⁷ were provided to and used in the West African countries affected by EBOV in 2014 to aid in outbreak management and control since the region had no prior experience with filovirus outbreaks. The

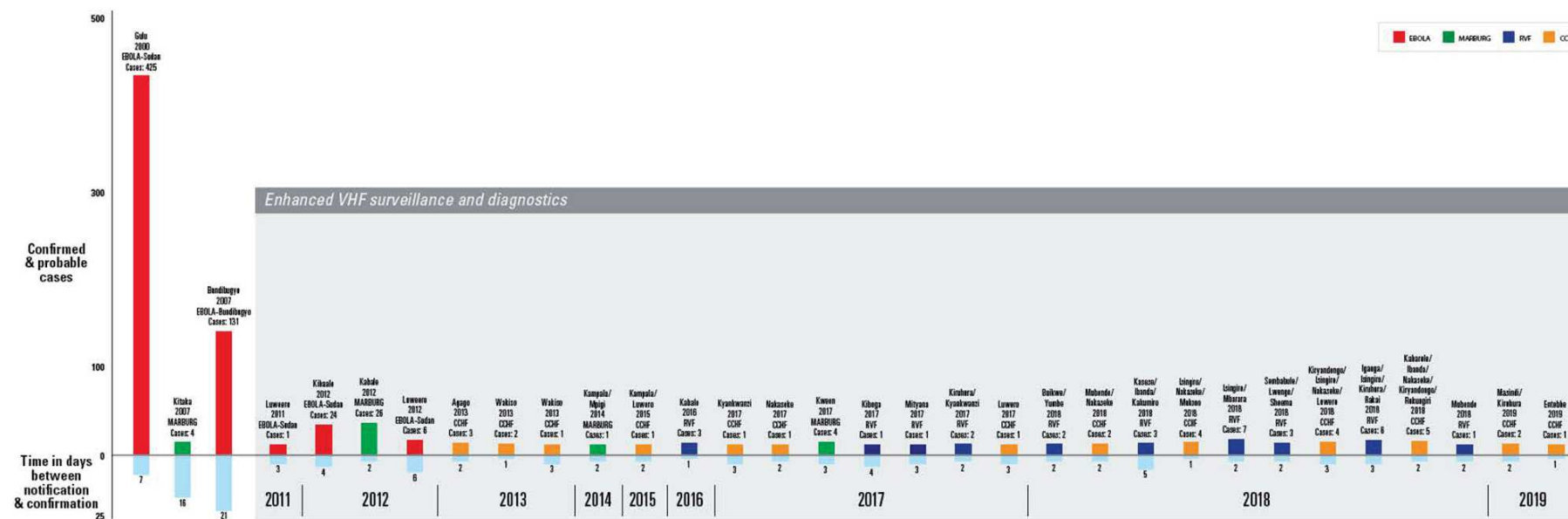


Figure 23: Impact of enhanced VHF surveillance in Uganda on the increase in outbreak detections and decrease in their magnitude and scope relative to the reduction in time from notification to laboratory confirmation (2011–2019). (unpublished figure)

program continues to serve as an Africa-wide resource providing technical assistance, expert training, and capacity building for ministries of Health, UN agencies, and international public health organizations. The UVRI VHF program highlights the importance of continued support and investment for dedicated public health surveillance and response programs for emerging zoonotic diseases and how these can significantly reduce the morbidity ($p = 0.001$) and mortality ($p = 0.01$) of VHF outbreaks.

Continuous VHF surveillance in Uganda allowed the identification and confirmation of 4 filovirus disease outbreaks during the second half of 2012. Following initial filovirus identification by conventional serological methods and qRT-PCR, sequence characterization of complete viral genomes from clinical samples confirmed the reemergence of SUDV and MARV in Uganda, and the first emergence of BDBV in DRC. Prior to 2012, BDBV had only been detected in a single outbreak in western Uganda in 2007⁷⁹. Furthermore, the sequence analysis indicated the reemergence of SUDV as 2 single but independent chains of transmission events in July and October 2012. Because these 4 filoviruses outbreaks occurred in such a short period of time, it was suspected that the increased emergence of filoviruses may be due to their changing genetic characteristics. Genetic analysis studies revealed, however, the sequences were not significantly different from previously detected and characterized filoviruses isolates circulating in the region. This increase in outbreaks, therefore, was likely due to the combination of improved surveillance and laboratory capacity, increased contact between humans and the natural reservoir of the viruses, and fluctuations in viral load and prevalence within this reservoir. The roles of these proposed explanations must be investigated further in order to guide appropriate responses to the changing epidemiological profile¹¹⁸.

Highlighting the success of the UVRI VHF surveillance and response program, the selected manuscripts range from the first re-emergence of SUDV in 2011 after a 10-year absence to the first confirmation of RVFV in nearly 50 years. This body of work illustrates how successful implementation of a broad VHF surveillance strategy and program can lead to successful and comprehensive outbreak detection and characterization of re-emerging VHFs. These 2 outbreaks describe the spectrum of response and control ranging from a single isolated case of EVD to multiple cases of RVF, including both humans and livestock. In addition to describing the seroprevalence of RVFV in Uganda and the genetic characterization of viral isolates, the KAP survey provided additional information regarding personal beliefs and practices regarding high-risk behaviors and occupations.

The 2016 outbreak of RVFV in Kabale District represents the first confirmed human cases of RVF in Uganda since 1968. In total, 4 laboratory-confirmed acute cases (AC1-4) and 2 probable deaths (PC1 and 2) were identified. The 3 acute cases found to be RVFV-positive by PCR (AC1, 2, and 4) were identified at health care facilities, with the 1 IgM-positive acute case (AC3) identified through laboratory testing of community members residing in affected villages. A multi-disciplinary team carried out a rapid and comprehensive outbreak investigation within 2 days of initial laboratory confirmation, including collection of human, livestock, and vector samples to identify additional cases and

possible sources of infection. Laboratory samples collected from 19 family and community members of confirmed and probable cases revealed 2 additional seropositive, convalescent RVF cases (CC1 and CC2), showing that RVFV has been circulating in Kabale District prior to detection of this outbreak.

A total of 83 livestock samples were also collected, with 8 (9.6%) positive by IgG serology, showing evidence of previous infection with RVFV. IgM serology was not performed to determine how many livestock were recently infected, but samples were tested by RT-PCR to identify any active infections. One caprine sample from the home village of AC1 was found to be RVFV-positive by RT-PCR, showing acute infection and suggesting that active RVFV transmission was occurring at the time of the investigation. In addition, a total of 6 different genera of mosquito vectors, representing 33 species/sub-species, were collected near the locations of the confirmed and probable cases. RVFV was detected in 3 of 298 (1%) pools tested by RT-PCR, confirming presence of RVFV-infected vectors and suggesting mosquito-borne virus transmission occurring at the time of the outbreak. RVFV presence in Uganda has previously been confirmed on multiple occasions in humans, livestock, and mosquitoes. Most notably, the virus had been detected in *Aedes africanus* and *Aedes circumluteolus* mosquitoes in 1955 in Lunyo near Entebbe; in 1960 in febrile patients near EAVRI, along with isolations from *Mansonia africana* and *Mansonia uniformis*; and again in 1963 in 2 febrile patients from the Entebbe area^{84, 119, 120}.

Multiple RVF epidemics have been documented in Uganda, the last (prior to 2016) occurring in 1968 and involving 7 individuals living in the Entebbe area, near what is now UVRI⁸⁴. These individuals lived near a forested area on the outskirts of Entebbe and presented to the EAVRI clinic within 1–5 days of onset of fever, headache, abdominal pain, vomiting, and chills. These symptoms were very similar to those of the patients presenting from the 2016 outbreak in Kabale. Since only 7 confirmed human cases were documented during the Entebbe outbreak, RVFV transmission was probably limited, indicating maintenance transmission or incidental transmission from enzootic maintenance rather than a widespread epizootic.

In contrast to epizootics in Kenya and Tanzania, which involved over 300 cases each, only 4 acute RVFV cases were identified in Kabale between March and July of 2016^{121, 122}. These identified cases likely represent more severe and complicated RVFV infections, and were identified because the patients sought care. Likely, the total number of infected persons in the region was much higher. The investigations revealed a low level of seropositivity, with only 2 of 19 samples containing detectable levels of IgG, and only 1 of the 4 acute cases having both IgM and IgG. No additional RT-PCR-positive human cases were detected, indicating that this outbreak was not part of a large, expansive epizootic like the ones that had occurred in neighboring countries. For comparison, overall human IgG seropositivity was 0.7–23% in Kenya, with a maximum local seroprevalence of 29%, and 5.2–11.7% in Tanzania, with a maximum local seroprevalence of 29.3%¹²³⁻¹²⁸.

The limited nature of the epizootic in Kabale District may be due in part to the district's geography. The region has many isolated hills and valleys, creating pockets of enzootic activity and isolated virus maintenance between mosquitoes and livestock. Humans may be only incidentally infected, either directly by mosquito vectors or by exposure to infected livestock. Another reason for the limited number of human RVFV cases may be the lack of vectors that both transmit RVFV and preferentially feed on humans.

Serial clinical samples were obtained from 3 of the 4 patients with acute, RT-PCR-positive, non-fatal RVF, allowing comprehensive laboratory monitoring of the antibody response and RNA viral loads in these individuals. Serum IgM titers were initially high ($\geq 1:6400$) in AC1 and AC2, suggesting a robust early immune response, although these samples were collected 8 and 15 days post symptom onset. IgG titers, on the other hand, were undetectable until the final sample collection on days 26 and 41. RT-PCR C_T values remained steady throughout the time course of infection despite evidence of a mounting immune response shown by rising antibody levels. No IgM was detected in the initial sample from AC4, but rising IgM and IgG titers were seen in the subsequent samples. RT-PCR C_T values decreased in this patient over time, indicating viral clearance.

These results are as expected for non-fatal RVFV infections and are similar to previously studied serially collected samples tested during an outbreak in Saudi Arabia⁸. The primary difference seen is the consistent C_T values throughout the course of infection in AC 1 and AC2 despite an immune response, suggesting some maintenance of RNAemia despite an active immune response. No fatal cases were studied in Kabale District, unlike in Saudi Arabia; fatal cases in that outbreak corresponded to very high initial C_T values that remained high throughout monitoring with no detectable immune response, which is indicative of fatal outcomes⁸. The fourth acute case (AC4) studied initially had high C_T values that steadily declined over time. A live RVFV isolate was only obtained from the first serially collected RT-PCR positive patient samples, indicating that the detectable RNA in subsequent samples may not equate to infectious virus, but rather be RNA lingering in serum within immune complexes or similar modifications. It is also possible that the prior handling, transport, and freezing and thawing of these clinical samples may have negatively affected the sensitive viral isolation assay, and thus it cannot be definitively stated that no infectious virus remains after 8 days of symptom onset.

The 2016 RVFV outbreak represents the 10th independent VHF outbreak detected and confirmed through the UVRI surveillance program. VHF outbreaks tend to become high-profile and generate widespread media attention, including nationwide health messaging and awareness, and so most districts have become sensitized to cases of severe disease that could be VHF. The surveillance system was enhanced in 2013 with the expansion of the national sample transportation network and establishment of the Public Health Operations Center, funded through the Global Health Security Agenda, to rapidly transport suspected clinical specimens from regional hubs to the national

reference laboratories¹²⁹. This rapid response demonstrates the significant public health impact of having established, ongoing, dedicated VHF surveillance to mitigate morbidity and mortality of endemic zoonotic infectious diseases.

Risk factors for RVFV seropositivity have been previously reported. A 2015 study in Kenya found that male sex, increased age, history of slaughtering livestock, history of malaise, and poor measured visual acuity were all factors for increased seropositivity^{125, 130}. Although the manuscript presented here found no association between sex and RVFV seropositivity after adjusting for other factors like occupation, there was an association with being a butcher (i.e., someone who cuts meat either at home or at a slaughterhouse) and RVFV seropositivity. Previous studies also found that drinking raw milk may be associated with RVFV seropositivity^{123, 131}, but no such association was found in this study, likely because few individuals (36; 5%) reported drinking raw milk. Anecdotally, individuals reported not drinking raw milk due to concerns about *Brucellosis* infection. No significant association between age and seropositivity was found, but interestingly, no persons younger than 20 years had evidence of RVFV infection. This may be because only 6 individuals below the age of 20 reported having close contact with livestock, with the majority in this age group reporting to be living full time at boarding school, greatly reducing their risk of exposure to potentially infected livestock.

The study suggests that RVFV transmission to humans in Kabale District is primarily due to exposure to the blood and body fluids of infected livestock, given that butchers and those handling raw meat were most likely to be RVFV-seropositive. Additionally, human seropositivity was found to be significantly associated with livestock seropositivity in each subcounty. Cattle density has also been previously associated with RVFV seropositivity in RVFV models¹³². In the study, significantly more cattle were seropositive (27%) than goats (7%) or sheep (4%). This difference could be due to mosquito feeding behavior, as mosquitoes tend to select large, ornamented species¹³³. Also, cattle tend to live longer than goats and sheep, and thus have more time to be exposed to infection. Furthermore, sheep and goats are usually kept indoors, especially at night, while cattle are rarely sheltered in Kabale District and are thus exposed to nighttime-biting mosquitoes. Other factors, such as the mosquito species involved, could have played a role in RVFV transmission in Kabale District. Mosquitoes collected after outbreak investigations were mostly animal-specific feeders rather than human-specific¹³, indicating that RVFV is primarily transmitted by mosquitoes within animal and human populations, but that humans are also infected from direct contact with infected animals.

Comparing these results with serological studies conducted in the neighboring countries of Kenya and Tanzania, where RVFV is endemic, provides some insight into the serosurvey findings. In a study in coastal area of Kenya from 2009–2011¹³⁴, RVFV seroprevalence in humans was lower (1.8%) than in this study. Similarly, the seroprevalence in humans was only 5.2% in Mbeya region in Tanzania¹³⁵, compared to 13% in this study. This is likely because samples were collected following a confirmed outbreak or uptick in inter-epidemic transmission, unlike in the Kenya study. However, seroprevalence was higher in domestic ruminants in another study in Garissa, Kenya (27.6%)¹³⁶, than in this

study (13%). Generally, seroprevalence in both animals and humans is expected to be higher in RVFV-endemic regions of Kenya and Tanzania than what was found in this study, but the risk factors identified were the same – mainly, contact with livestock.

In addition to the serosurvey, a KAP survey was completed to identify knowledge gaps in order to inform intervention measures and develop health education and communication materials on RVF prevention. This study found that in the agricultural community of Kabale District, most survey participants had heard of RVF, although few human cases had ever been identified in Uganda, and none had been identified since the 1960s. Most of these participants received their information regarding RVF from the radio. This may have been in part due to local efforts to increase RVF awareness recently through radio announcements following the recent detection of the human cases.

Although 90% of participants had heard of RVF, many did not recognize the most common signs and symptoms of RVF in humans and animals. The most common symptom stated by participants in both humans and animals was bleeding; however, bleeding is a rare symptom of RVF. Survey participants may be confusing RVF symptoms with symptoms of MARV infection, which more often presents with hemorrhagic symptoms, since a MARV cluster was identified in Kabale District in 2012³². These findings are similar to previous findings in other studies done in Kenya and Tanzania, despite differences in this study populations^{89, 92}. Compared to similar studies in Kenya and Tanzania, this study population had higher education; nearly 55% of the participants had secondary educations compared to 88% of participants having no formal education in the Kenya study (Abdi et al)⁸⁹. Further, Kenya has had more documented RVF outbreaks than Uganda, with a particularly large outbreak in 2006–2007 that significantly impacted the Kenyan pastoralist economy⁶¹. However, even in the Abdi study, most individuals (92%) thought that hemorrhage was an RVF symptom and less than 40% recognized headache, myalgias, and visual changes as RVF symptoms in humans. This study and the Abdi study suggest that even in settings where RVF is endemic or where formal education is prevalent, misconceptions about RVF exist.

Symptom and sign recognition in animals and humans is imperative to prevent the spread of RVFV, especially in a community such as Kabale, where 60% of survey participants own animals. During the 2007 RVF outbreak in Sudan, Hassan et al. noted that an RVF outbreak was only recognized after human cases were identified, indicating that improved awareness of the disease in animals should be emphasized⁶⁸. When at-risk communities have heightened awareness of RVF symptoms in animals, they may be able to improve surveillance and identify an outbreak early. Jost et al. conducted focus groups with pastoralists in Kenya and Tanzania during the 2006–2007 RVF outbreak and found that pastoralists not only recognized changes in weather patterns and mosquito swarms, but also recognized RVF signs among animals and humans⁹¹.

Many participants (47%) of the KAP study said that they did not have an understanding of RVFV transmission. Nearly a third (31%) of all participants thought that RVFV could be transmitted by bodily contact and 66% of farmers thought that RVFV could be transmitted by animal contact. Additionally, mosquito bites were an under-recognized mode of RVFV transmission (this was also noted in the Abdi study)⁸⁹. Interestingly, in another study in Kenya⁹⁰ by Owange *et al*, pastoralists felt that mosquitoes were a very important risk factor for RVFV transmission in cattle⁹⁰. Although this study demonstrated that participants did not consider mosquitoes to be an important risk factor for RVF, participants said that they frequently slept under mosquito nets, likely due to concern for malaria. Improving education regarding other mosquito-borne diseases, including yellow fever and RVF, can further stress the importance of prevention against mosquito bites.

Studies of EVD and MVD survivors in Uganda and elsewhere have demonstrated that many survivors experience stigma within their community¹³⁷. Unlike EBOV or MARV, RVFV cannot be transmitted from human to human. This study demonstrated that stigma is not commonly associated with RVF and that most community members would interact with survivors and welcome them into their community.

The objective of this study was to conduct a rapid, timely KAP study in order to inform an educational campaign, and as a result this study had several limitations. Because the sampling strategy was convenience-based, the survey participants may not accurately represent the true population in Kabale District. However, given that over 650 participants in the region were interviewed, focusing in part on high risk populations, this study is still very valuable. Many of the questions were yes/no or multiple choice in format, and no focus groups were convened, so the results are mainly descriptive. Some of the questions regarding RVF symptoms were not specific to RVF and could overlap with other diseases, including MVD.

Following the analysis of the survey, the MAAIF, Uganda MOH, UVRI, and CDC worked together to develop RVF educational materials targeting abattoir workers, farmers, herdsman, and other community members (Figure 24). The posters emphasized that signs and symptoms of RVF more commonly include diarrhea, vomiting, and fever. These posters were created to target a low-literacy rate audience using culturally appropriate messaging highlighting key findings from the KAP study, including disease transmission, signs and symptoms of RVF in humans and animals, and safe cooking practices¹³⁸. For example, a poster targeting farmers and herdsman contains images of symptomatic animals and humans and advises that farmers and herdsman should contact veterinary staff if their animal is ill. These materials have been translated to French for use in Niger after the identification of RVF cases in 2016¹³⁹. The CDC Viral Special Pathogens website also serves as a resource for information about previous outbreaks and RVF symptoms and diagnosis¹⁴⁰.

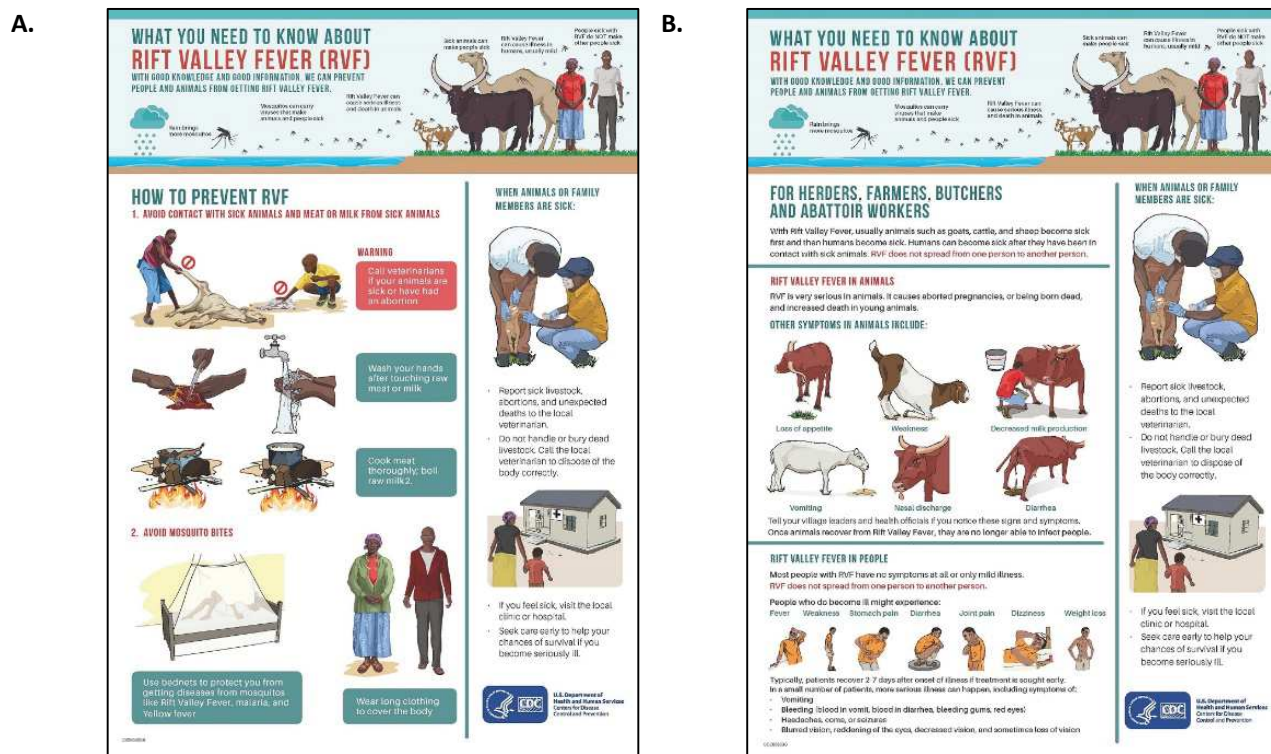


Figure 24: RVF health education materials designed based on KAP survey data collected in Kabale District in 2016. **A.** RVF health information for the general population. **B.** RVF health information for herders, farmers, butchers, and abattoir workers.¹

5 Conclusions and Perspectives:

The detection of the multiple outbreaks and subsequent studies described in this thesis illustrate how the development and use of molecular techniques can be applied to detect VHF outbreaks, leading to rapid response and outbreak investigation and further to characterize the epidemiological and genetic features of these viruses. This thesis also illustrates how these diagnostic and epidemiological investigation methods have been routinely validated through real-world VHF surveillance and laboratory diagnostic testing. The scientific studies outlined above highlight significant knowledge that was gained and described in each objective at the time of publication of the corresponding manuscripts.

Objective 1 describes the first outbreak of RVFV identified in Saudi Arabia and Yemen in 2000. Prior to this, RVF outbreaks were not known to occur outside of the African continent. It was not known when or how this virus crossed over to the Arabian Peninsula, so thorough investigations and genetic characterization of the virus from this outbreak was necessary to address this question. Thorough genetic characterization of RVFV isolates described in Manuscript 1

showed that the virus found in Saudi Arabia and Yemen was the same as a previously described strain circulating in East Africa during a 1997–1998 epizootic. These data also suggested that animal importation from the African continent into Saudi Arabia likely contributed to RVFV in that region in 2000. RVFV-infected livestock importation and resulting emergence of RVFV in previously RVFV-free regions was also seen on Mayotte islands beginning as early as 2004¹⁴¹ and again more recently in 2018.

Objective 2 addresses the development of a new standardized, broadly applicable molecular diagnostic method to detect diverse strains of RVFV. The 1997–1998 East African RVFV epizootic and the 2000 RVFV outbreak in Saudi Arabia and Yemen underscored the need for a real-time reverse transcriptase-based molecular diagnostic technique that could detect all known RVFV isolates and could be used in future RVFV outbreaks. At the time, real-time RT-PCR molecular diagnostics were becoming the standard for clinical diagnostics. Prior to these assays, standard PCR and gel-based detection techniques were used, the sensitivity and specificity of which were not fully known. Manuscript 2 described the development of a highly sensitive and specific molecular technique using RVFV genetic data spanning from 1944 to 2000 and showing an approximately 5% difference at the nucleotide level. Using clinical material from the 1997–1998 and 2000 RVFV outbreaks, this new diagnostic was developed and validated, and has since become a widely used tool for RVFV detection. It is now considered the gold standard molecular diagnostic assay for RVFV.

Objective 3 highlights the development of a new and innovative surveillance system in Uganda to quickly identify, confirm, and respond to new VHF outbreaks. Prior to establishing this new comprehensive national VHF surveillance system, Uganda had no formal method of standardized VHF surveillance or diagnostic capacity to perform laboratory testing if a suspected VHF case was identified. Testing suspect VHF samples had to be performed by international laboratories in the United States, Europe, or South Africa, drastically delaying the results that may be urgently needed for further action and intervention. The establishment of this system was meant to address this gap in both technical surveillance capacity and laboratory diagnostic capacity so both could be done within Uganda and dramatically reduce the time from case identification to confirmation. Manuscript 3 describes the first confirmed case detected using this newly established VHF surveillance system and laboratory diagnostics, which was a single case of SUDV. This turned out to be the initial and presumed index case, and only the second published account of a single EVD case. Prior to this, the last outbreak of SUDV occurred 10 years earlier in 2000, though undetected EVD cases may have occurred. The VHF surveillance system and testing laboratory helped address this gap in capacity and show that more VHF outbreaks may be occurring than previously thought because they were not detected previously due to poor surveillance.

Objective 4 describes the effectiveness of the Uganda VHF surveillance system following 6 years of outbreak detection and investigations. After the first EVD case detection in 2011, 15 independent VHF outbreaks were detected and laboratory confirmed by this system, including multiple filovirus and CCHFV outbreaks and an RVFV outbreak. In

order to determine if this new system was significantly effective in both detecting and helping to control outbreaks compared to historical outbreaks pre-surveillance, multiple factors and indices were compared from VHF outbreaks that occurred between 2011–2017 and the larger outbreaks that had occurred between 2000–2007. Manuscript 4 illustrates that by all measures, the surveillance system statistically reduced the time from suspect case identification to confirmation, shortened duration of the outbreak, and reduced outbreak severity (number of overall cases), secondary transmissions, and morbidity and mortality. This was the first time a comprehensive surveillance system had been shown to make such an impact; even though more outbreaks can be detected, prompt identification and response significantly lowered their negative outcome. The system and the materials and methods used to establish it have since been used in the 2014–2016 West Africa EVD outbreak, informing response to VHF outbreaks and providing technical materials during the largest EVD outbreak on record.

Objective 5 compares the genetic sequences of multiple filovirus isolates that were identified and obtained from VHF outbreaks detected and investigated by the Uganda VHF surveillance program. Following an unprecedented 4 independent filovirus outbreak detections, laboratory confirmations, and investigations all in 2012, the question arose why so many outbreaks were occurring in such a short period of time. One hypothesis was that the viruses were somehow becoming more transmissible either through a genetic change or more easily transmitted from their zoonotic source to humans. Manuscript 5 shows that detailed genetic analysis and comparisons to historical filovirus strains and recent filovirus isolates from Uganda revealed no significant genetic changes in these viral isolates that would affect transmissibility, pathogenicity, or virulence. Other factors must have played a role in increasing human infections through zoonotic sources or from other individuals prior to initial detection through routine surveillance. Alternatively, the increased number of outbreaks could simply be due to improved detection of the initial cases, showing that the surveillance system was performing as expected in detecting VHFs and initiating a rapid and effective response.

Objective 6 describes the detection, laboratory confirmation, and subsequent research studies of the first RVFV human cases in Uganda in nearly 40 years. Manuscript 6 describes the initial case detections and epidemiological investigations, as well as describing the animal and mosquito vector assessments conducted in 2016. Since RVFV was not known to be actively circulating in Uganda, comprehensive investigations were imperative to identify potential sources and vectors and to determine if the virus was newly introduced. Additionally, genetically characterizing this newly identified RVFV isolate was crucial to identifying whether it was imported from other regions or had been endemic in Uganda. We determined that these new cases were likely infected locally and were not travel-related. We also determined after genetic sequencing that the RVFV isolate causing the infections was most closely related to the East African 2006–7 epizootic strain that occurred primarily in Kenya and Tanzania. The information gained from this investigation was also valuable in that it showed infected mosquitoes as well as infected livestock, showing we had identified the outbreak during a time of active circulation thus demonstrating that RVFV was established in Uganda.

Manuscripts 7 and 8 detail seroprevalence and KAP surveys that were conducted immediately following the initial RVFV cases. Since no laboratory-confirmed human cases of RVFV had been detected in Uganda in nearly 40 years, a thorough investigation was needed to identify any unknown acute human cases as well as determine the prevalence of previous RVFV infection. In addition, since most people in this region had not experienced or were unfamiliar with RVF disease, their familiarity and knowledge needed to be assessed to better inform and educate the population for preventing RVFV and reporting future cases. Both studies provided an immense amount of data showing some previous RVFV circulation and infection in this region. In addition, it revealed the current knowledge level of the residents so additional action, education, and prevention measures could be developed. RVFV seroprevalence surveys had not been done previously in Uganda, so this study laid the groundwork for additional knowledge and active research that has since continued and led to identification of many additional RVFV cases in Uganda since this 2016 outbreak

6 Personal Perspective:

The VHF surveillance program continues to utilize the range of molecular serological diagnostic assays described, showing the successful integration and range of my scientific and professional development from the laboratory to field-based epidemiology and investigation. My outbreak and surveillance work in Uganda, as well as 20 years of experience in public health and laboratory research, comprise a comprehensive and interrelated set of professional activities contributing to a diverse and multidisciplinary array of contributions to public health and scientific understanding.

This professional experience has also allowed me to build an extensive network of scientific and public health collaborators and partnerships in which to expand and diversify the work described above. Specifically, this has led me to develop a professional collaboration with CIRAD and the University of Montpellier focused on further characterizing zoonotic viral diseases by improving animal and human surveillance methods, validating new laboratory diagnostic methods, and characterizing entomological and environmental factors that contribute to the emergence and re-emergence of VHFs in Africa. This further work will be accomplished by leveraging the specific strengths and expertise of CIRAD and University of Montpellier with the US CDC and governmental and non-governmental partners in Africa to work towards the common objective of understanding, monitoring, and reducing potential epidemics of zoonotic viral diseases.

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8 Manuscripts:

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